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## Immunologic Markers in the Differential Diagnosis of Non-Hodgkin's Lymphomas\*

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**Summary.** To improve the classification of the phenotypes of the various types of non-Hodgkin's lymphoma (NHL), 250 cases of NHL were analyzed with immunologic and enzyme cytochemical techniques. The results confirmed previous findings. Chronic lymphocytic leukemia of B-cell type (B-CLL) is characterized by a small amount of surface immunoglobulin (SIg), a predominance of C3d receptors, a large number of mouse erythrocyte receptors, and a low T-cell content. Chronic lymphocytic leukemia of T-cell type (T-CLL) has at least two subtypes: one shows a dot-like reaction product in cells stained with acid nonspecific esterase and the other does not. In prolymphocytic leukemia, there is a constantly high percentage of SIg-positive cells and a large amount of SIg on each positive cell; C3b receptors usually preponderate over C3d receptors; and there is a large number of IgG-Fc receptors. The surface marker phenotype of hairy-cell leukemia is similar to that of prolymphocytic leukemia except that hairy cells are devoid of C3 receptors and usually show a high density of IgM-Fc receptors. T-zone lymphoma usually contains both T cells and B cells. The T cells are capable of binding sheep erythrocytes only at 4 °C and can be identified cytologically as the tumor cells. In contrast, the B cells stem from residual follicles, which are often present in T-zone lymphoma at the time of the first biopsy. Three types of lymphoplasmacytic/cytoid lymphoma (LP immunocytoma) are distinguished on the basis of morphologic features. The marker constellation of the lymphoplasmacytic subtype resembles that of centroblastic-centrocytic lymphoma. The lymphoplasmacytoid subtype and borderline cases between this subtype and B-CLL show the same markers as does B-CLL. The third subtype of LP immunocytoma, the polymorphic subtype, differs in its marker profile from all other types of NHL. The three types of lymphoma derived from germinal center cells resemble each other in the expression of nearly equal numbers of C3b and C3d receptors and a low percentage of IgG-Fc receptors. Centrocytic lymphoma is distinguished from centroblastic-cen-

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trocytic lymphoma by a large proportion of cells bearing SIg and C3 receptors and by the absence, or small proportion, of T cells and cells rosetting with mouse erythrocytes. Centroblastic lymphoma shows a marker profile that is similar to that of centroblastic-centrocytic lymphoma. The Burkitt type of lymphoblastic lymphoma shows a unique marker profile, with a high percentage of SIg-positive cells and no other markers. Analysis of lymphoblastic lymphoma of the convoluted-cell type (including cases of acute lymphoblastic leukemia with a focal acid phosphatase reaction) revealed four phenotypes. Cases with the first phenotype show C3 receptors (usually both subtypes) and a lack of sheep erythrocyte receptors. In cases with the second phenotype, the cells express both C3 receptors and sheep erythrocyte receptors. Cases with the third phenotype lack C3 receptors but contain cells rich in receptors for sheep erythrocytes that bind at 37 °C. Nearly all of the cases with these three phenotypes are devoid of acid nonspecific esterase. Cases with the fourth phenotype lack C3 receptors, exhibit sheep erythrocyte receptors that bind only at 4 °C, and show a focal acid nonspecific esterase reaction. Eight of nine cases of immunoblastic lymphoma showed SIg and were thus identified as B-cell derived. The ninth case was of T-cell type, as indicated by the capacity of the tumor cells to form rosettes with sheep erythrocytes.

**Key words.** Non-Hodgkin's lymphomas – Surface markers – Enzyme markers

ML lymphocytic
B-CLL
T-CLL
Prolymphocytic leukemia
Hairy-cell leukemia
Mycosis fungoides and Sézary's syndrome
T-zone lymphoma
ML lymphoplasmacytic/lymphoplasmacytoid
ML centrocytic
ML centroblastic-centrocytic
MU centroblastic
ML lymphoblastic
Burkitt type
Convoluted-cell type
Unclassified
ML immunoblastic

**Table 1.** Kiel classification of non-Hodgkin's lymphomas

## Introduction

The Kiel classification of non-Hodgkin's lymphomas (NHL; Table 1) was established mainly on the basis of cytomorphologic and immunochemical studies (Stein et al. 1972; Lennert et al. 1975a, b; Stein 1976). To improve this basis we have performed a multiple marker analysis of the various NHL types since 1976. Preliminary results of this analysis were reported in a number of previous papers (Stein 1978; Stein et al. 1978, 1979; Stein and Tolksdorf 1979; Tolksdorf et al. 1980).

The aim of the present paper is to give a detailed survey of our findings on a total of 250 cases of NHL. We will not attempt to compare our findings with data

from the literature, because our different classification systems so that of this type would also go beyond the relationship between the entities of the NHL was handled in detail recently (Tolksdorf et al. 1980).

## Material and Methods

### Source of Material

The source and handling of the biopsy material (Tolksdorf et al. 1980).

### Surface and Enzyme Phenotyping

Table 2 shows the markers used. The assay for SIg (Tolksdorf et al. 1980). Acid phosphatase staining was performed with the following reagents: Acid nonspecific esterase staining was performed with the following reagents:

## Results and Discussion

We related the findings obtained in the NHL types and obtained the following results:

### Chronic Lymphocytic Leukemia of the B-cell Type

As Fig. 1 shows, the number of SIg-positive cells was usually high in chronic lymphocytic leukemia of the B-cell type. The number of SIg-positive cells was usually high in chronic lymphocytic leukemia of the B-cell type, but also varied greatly from the lymph node biopsy material.

Highly characteristic of B-CLL is the presence of C3b receptors, with a ratio of 3 to 1 of C3b receptors by a high percentage of cells.

The number of cells bearing IgG receptors was usually low. This probably reflects the detection assay used. This probably reflects the detection assay used.

The T-cell content was usually low. This probably reflects the detection assay used. This probably reflects the detection assay used.

of cells bearing SIg and C3 receptors, of T cells and cells rosetting with lymphoma shows a marker profile that is characteristic of lymphoma. The Burkitt type of lymphoma shows a marker profile, with a high percentage of cells bearing SIg receptors. Analysis of lymphoblastic lymphomas (usually both subtypes) and a lack of rosetting with the second phenotype, the cells express C3b receptors. Cases with the third phenotype are cells rich in receptors for sheep erythrocytes. Cases with these three phenotypes lack receptors that bind only at 4 °C, and do not show a positive reaction. Eight of nine cases of B-cell lymphoma were thus identified as B-cell lymphoma, as indicated by the capacity of the cells to rosette with erythrocytes.

#### – Surface markers – Enzyme markers

**Table 1.** Kiel classification of non-Hodgkin's lymphomas

lymphomas (NHL; Table 1) was established by histologic and immunochemical studies (Stein et al. 1976). To improve this basis we have performed a survey of various NHL types since 1976. Preliminary results are in a number of previous papers (Stein et al. 1979; Tolksdorf et al. 1980). In this paper we present a detailed survey of our findings on B-cell lymphoma to compare our findings with data

**Table 2.** Surface and enzyme markers

SIg
C3b receptors
C3d receptors
Mouse erythrocyte receptors
IgG-Fc receptors
IgM-Fc receptors
Sheep erythrocyte receptors
HLA-DR
Acid phosphatase
Acid nonspecific esterase

from the literature, because our data and those of other authors are based on different classification systems so that they are not really comparable. A comparison of this type would also go beyond the limits of this survey. Moreover, the relationship between the entities of the Kiel classification and those of other classifications was handled in detail recently (Lennert and Mohri 1978).

### Material and Methods

#### Source of Material

The source and handling of the biopsy material and blood samples are described in detail elsewhere (Tolksdorf et al. 1980).

#### Surface and Enzyme Phenotyping

Table 2 shows the markers used. The assays for surface markers are described elsewhere (Stein 1978; Tolksdorf et al. 1980). Acid phosphatase activity was demonstrated with the method of Leder (1967). Acid nonspecific esterase staining was performed as described recently (Tolksdorf and Stein 1979).

### Results and Discussion

We related the findings obtained in the marker studies to the morphology of the NHL types and obtained the following results.

#### Chronic Lymphocytic Leukemia of B-cell Type (B-CLL)

As Fig. 1 shows, the number of SIg-positive cells varied greatly from case to case of chronic lymphocytic leukemia of B-cell type (B-CLL). However, the percentage of SIg-positive cells was usually higher in the blood than in the suspension prepared from the lymph node biopsy. The SIg density (not shown in Fig. 1) was usually low, but also varied greatly from case to case.

Highly characteristic of B-CLL were a preponderance of C3d receptors over C3b receptors, with a ratio of 3 to 1, and the expression of mouse erythrocyte receptors by a high percentage of cells.

The number of cells bearing IgG-Fc receptors varied greatly, depending on the detection assay used. This probably reflects the varying density of IgG-Fc receptors on the tumor cells of B-CLL.

The T-cell content was usually low, a finding that proved to be helpful in differentiating B-CLL from certain cases of lymphoplasmacytic/cytoid lymphoma.

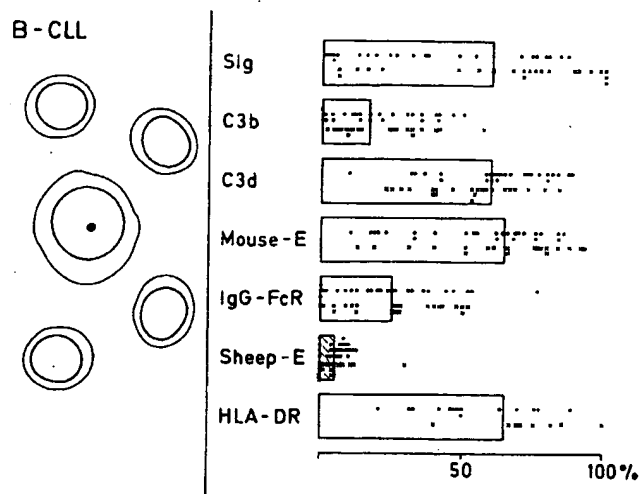


Fig. 1. Chronic lymphocytic leukemia of B-cell type (B-CLL). On the left, a symbolic representation of the morphology of the cells that proliferate in this type of lymphoma. On the right, the results of marker studies are plotted. The abscissa shows the percentage of positive cells obtained for the different markers. The dots symbolize the values obtained in lymph node suspensions and the x's symbolize the values obtained in blood cell concentrates. The length of each rectangle indicates the median value.

#### Chronic Lymphocytic Leukemia of T-Cell Type (T-CLL)

In five of the cases of chronic lymphocytic leukemia in our series, the cells were capable of binding sheep erythrocytes at 4 °C (but not at 37 °C) and lacked SIg, receptors for mouse erythrocytes, and C3 receptors. The cells from four of these cases exhibited a focal or dot-like acid esterase reaction product and lacked IgG-Fc receptors, as do the cells of the normal T-cell subpopulation with a helper function. The cells of the fifth case were characterized by a very large amount of weakly basophilic cytoplasm, which contained azurophil granules in 20–30% of the cells. This case showed IgG-Fc receptors but no acid esterase reactivity. Its phenotype thus corresponds to that described for the T-cell subpopulation with a suppressor function.

#### Chronic Lymphocytic Leukemia with an Anomalous Phenotype

Our series contained another case with an exceptional marker profile. In this case, 85% of the cells bound sheep erythrocytes at 4 °C (but not at 37 °C) and reacted with an anti-T-cell serum (Rodt et al. 1975), but 35% of the cells reacted with F(ab)<sub>2</sub> fragments directed against IgM, IgD, or  $\kappa$ . C3d receptors were expressed by 91% of the cells and IgG-Fc receptors by 72%. Strong, focal acid phosphatase and acid esterase reactions were found in 70% of the cells. Thus, the phenotype of this case does not fit into any of the known T- or B-cell categories.

#### Prolymphocytic Leukemia

In contrast to B-CLL, all of the 11 cases of prolymphocytic leukemia investigated (Fig. 2) showed a high percentage of SIg-positive cells and a high SIg density. Al-

#### Immunologic Markers in the Different

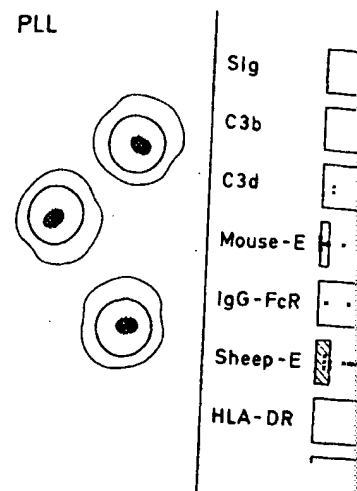


Fig. 2. Prolymphocytic leukemia (PLL). F

though the number of cells bearing the number of C3b receptor-positive cells in most instances. The receptors was usually very low and usually expressed by a larger percentage in B-CLL. In a minority of cases of number of cells bearing IgM-Fc receptors. In agreement with the findings of 11 cases of prolymphocytic leukemia, phatase activity that could not be in cases of prolymphocytic leukemia is receptors for sheep erythrocytes.

In our series there was one exception of prolymphocytic leukemia cells but erythrocyte receptors. The cells of this case (EAC), or fluid phase C3, but, surpr

#### Hairy-cell Leukemia

Figure 3 illustrates the marker profile of hairy-cell leukemia. Hairy cells were characterized by high affinity IgG-Fc receptors. In prolymphocytic leukemia. The only exception of prolymphocytic leukemia and absence of C3 receptors in hairy cell leukemia.

Much of the data in the literature on hairy cells is contradictory. This may be due to the presence of high affinity receptors. The presence of high affinity

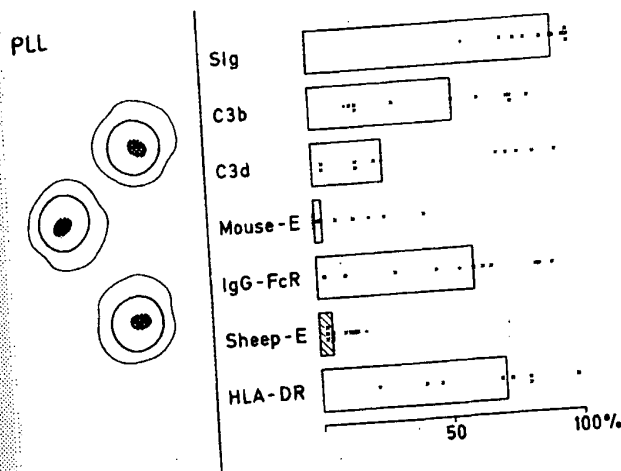


Fig. 2. Prolymphocytic leukemia (PLL). For explanations see Fig. 1

(CLL). On the left, a symbolic representation of lymphoma. On the right, the results of marker age of positive cells obtained for the different lymph node suspensions and the x's symbolize the median value of each rectangle indicates the median value

#### Type (T-CLL)

leukemia in our series, the cells were characterized (but not at 37 °C) and lacked SIg, receptors. The cells from four of these cases reacted with a helper product and lacked IgG-Fc receptors. The cells from the other four cases reacted with a helper product and lacked IgG-Fc receptors. The cells from the other four cases reacted with a helper product and lacked IgG-Fc receptors. The cells from the other four cases reacted with a helper product and lacked IgG-Fc receptors.

#### Unusual Phenotype

exceptional marker profile. In this case, cells reacted at 4 °C (but not at 37 °C) and reacted with IgG, but 35% of the cells reacted with IgG, or κ. C3d receptors were expressed by 72%. Strong, focal acid phosphatase activity was present in 70% of the cells. Thus, the phenotype was not known T- or B-cell categories.

of prolymphocytic leukemia investigated positive cells and a high SIg density. Al-

though the number of cells bearing C3 receptors varied greatly from case to case, the number of C3b receptor-positive cells exceeded the number of C3d receptor-positive cells in most instances. The percentage of cells bearing mouse erythrocyte receptors was usually very low and never exceeded 50%. IgG-Fc receptors were usually expressed by a larger percentage of cells in prolymphocytic leukemia than in B-CLL. In a minority of cases of prolymphocytic leukemia we found a moderate number of cells bearing IgM-Fc receptors.

In agreement with the findings of other authors (Catovsky et al. 1974), in three of 11 cases of prolymphocytic leukemia we found strong, granular acid phosphatase activity that could not be inhibited by tartrate. Remarkably, none of the cases of prolymphocytic leukemia in our series expressed T-cell antigens or receptors for sheep erythrocytes.

In our series there was one exceptional case that showed the cytologic features of prolymphocytic leukemia cells but lacked SIg and did not express sheep erythrocyte receptors. The cells of this case also did not bind membrane-bound C3 (e.g. EAC), or fluid phase C3, but, surprisingly, they did bind fluid phase C4.

#### Hairy-cell Leukemia

Figure 3 illustrates the marker profile that we obtained for 17 cases of hairy-cell leukemia. Hairy cells were characterized by the expression of large amounts of SIg and high affinity IgG-Fc receptors. In this respect, hairy cells resemble the cells of prolymphocytic leukemia. The only significant difference between the marker profile of prolymphocytic leukemia and that of hairy cell leukemia was the constant absence of C3 receptors in hairy cells.

Much of the data in the literature on the presence of light chain types on hairy cells is contradictory. This may be due to the expression of high affinity IgG-Fc receptors. The presence of high affinity IgG-Fc receptors is a constant and very

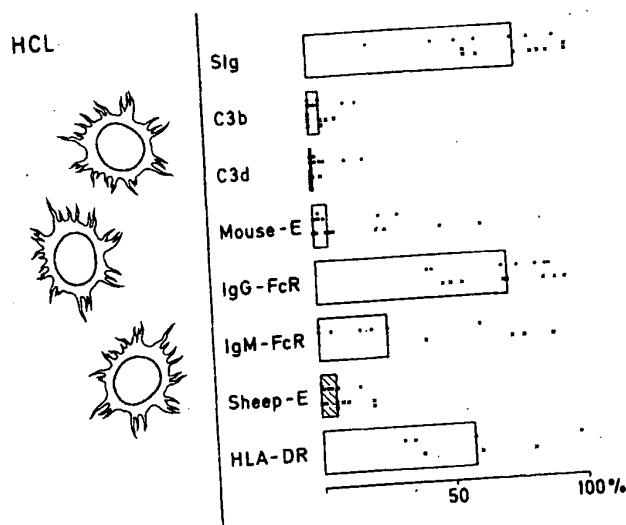


Fig. 3. Hairy-cell leukemia (HCL). For explanations see Fig. 1

characteristic feature of hairy cells. These high affinity IgG-Fc receptors were apparently responsible for the repeatedly false antibody labeling results, which showed both  $\kappa$  and  $\lambda$  on hairy cells.

When  $F(ab)_2$  fragments were used for labeling, all cases of hairy-cell leukemia proved to be clearly monotypic in light chain expression. The SIg density, however, usually remained high. This is in line with findings of others (e.g., Rieber et al. 1977).

The falsely positive demonstration of C3 receptors on hairy cells was apparently due to the high affinity IgM-Fc receptors present in hairy cells because IgM and complement-coated erythrocytes were used as indicator cells. By changing the experimental conditions of the assay system (omitting pelleting of the hairy cells and EAC by centrifugation) we were able to confirm the results of Burns et al. (1977) that hairy cells bind IgM-EAC via the IgM molecule and not via C3 fragments. We have also found that hairy cells are not stained by the anti-C3 receptor serum produced in our laboratory (Gerdes et al. 1980).

The acid esterase reactivity of hairy cells proved to be a useful marker. Hairy cells of all but one case showed a semicircular, granular acid esterase reaction product, as reported recently (Tolksdorf and Stein 1979). This acid esterase reactivity was also present in most of the cases lacking tartrate-resistant acid phosphatase.

#### T-Zone Lymphoma

All five cases of T-zone lymphoma showed a high percentage of sheep erythrocyte rosette-forming cells (Fig. 4). The vast majority of the cells of all cases were able to form sheep erythrocyte rosettes only at 4 °C, suggesting that the sheep erythrocyte-forming cells were closely related to peripheral T cells.

#### T-zone lymphoma

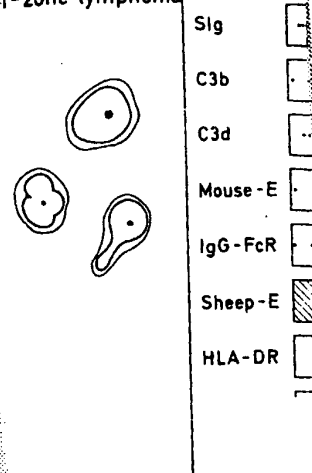


Fig. 4. T-zone lymphoma. For explanation see Fig. 1

In three cases a significant number of cells bore receptors for both C3b and C3d as B cells also bore receptors for IgG-FcR.

Morphological analysis of the sheep erythrocyte rosette-forming cell fraction present in varying percentages of the cases showed a wide range of terms of light-chain expression.

#### Lymphoplasmacytic/cytoid Lymphoma

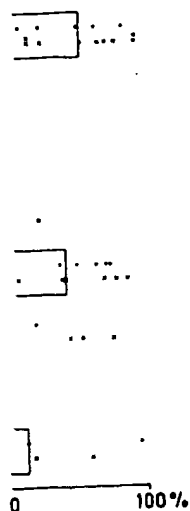
In contrast to B-CLL, which is a plasmacytoma, which is a proliferation of B cells (plasmacytoid cells) can be identified by the presence of cytoplasmic Ig (CIg) when stained with anti-Ig.

LP immunocytoma is not a homogeneous morphologically (Fig. 5).

The lymphoplasmacytoid subtype of lymphoma and some plasmacytoid and of borderline cases between B-CLL and B-CLL. The ratio of cells binding mouse erythrocytes in B-CLL.

The lymphoplasmacytic subtype of lymphoma is characterized by a mixture of different cells. The plasmacytoid type. This subtype showed a relatively low percentage of cells





see Fig. 1

high affinity IgG-Fc receptors were false antibody labeling results, which

abeling, all cases of hairy-cell leukemia n expression. The SIg density, however, 1 findings of others (e.g., Rieber et al.

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#### T-zone lymphoma

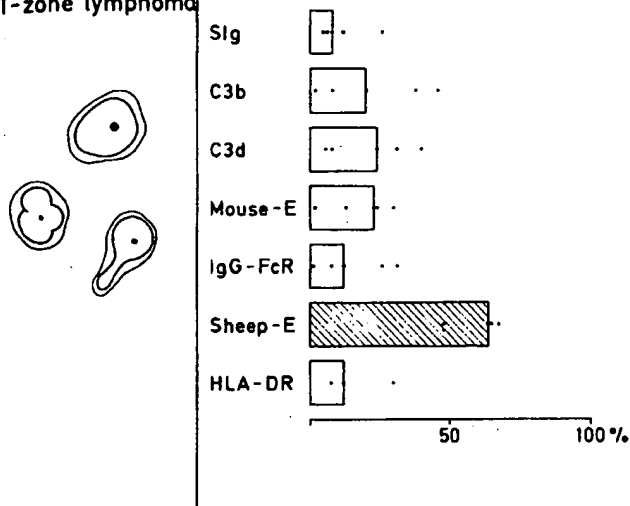


Fig. 4. T-zone lymphoma. For explanations see Fig. 1

In three cases a significant number of B cells were also present. They expressed receptors for both C3b and C3d and receptors for mouse erythrocytes. Some of the B cells also bore receptors for IgG-Fc.

Morphological analysis of the rosette-forming cells clearly indicated that the sheep erythrocyte rosette-forming cells were the neoplastic ones. Moreover, the B-cell fraction present in varying percentages in all cases proved to be polyclonal in terms of light-chain expression.

#### Lymphoplasmacytic/cytoid Lymphoma (LP Immunocytoma)

In contrast to B-CLL, which is a proliferation of nonsecretory B cells, and to plasmacytoma, which is a proliferation of secretory B cells, LP immunocytoma is defined as a mixed proliferation of nonsecretory and secretory B cells. The secretory B cells (plasmacytoid cells) can be identified by their content of easily detectable cytoplasmic Ig (CIg) when stained with the immunoperoxidase method.

LP immunocytoma is not a homogeneous entity. Three subtypes can be distinguished morphologically (Fig. 5).

The lymphoplasmacytoid subtype (see I in Fig. 5) shows a predominance of lymphocytes and some plasmacytoid cells. The marker constellation of this type and of borderline cases between B-CLL and the lymphoplasmacytoid subtype resembled that of B-CLL. The ratio of C3d to C3b receptors was 3:1, the percentage of cells binding mouse erythrocytes was high, and the T-cell content was low, as in B-CLL.

The lymphoplasmacytic subtype of LP immunocytoma (see II) is characterized by a mixture of different cells. The pathognomonic cell is a plasma cell of the Marschalkó type. This subtype showed nearly equal numbers of C3b and C3d receptors, a relatively low percentage of cells binding mouse erythrocytes, and a relatively

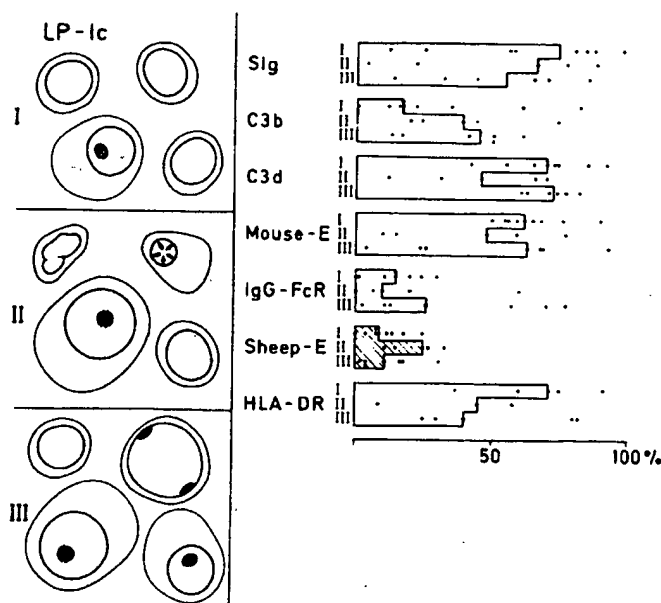


Fig. 5. Lymphoplasmatic/cytoid lymphoma (LP immunocytoma, LP-Ic). For explanations see Fig. 1

large number of T cells. This marker profile is similar to that of centroblastic-centrocytic lymphoma.

The polymorphic subtype (see III) is defined morphologically as a mixture of lymphocytes or lymphoid cells and plasmacytoid cells, interspersed with a large number of blast cells. The marker profile is not similar to any other pattern.

#### Lymphomas Derived from Germinal Center Cells

We distinguish three types of germinal center cell lymphoma.

##### Centrocytic Lymphoma

The first type of malignant lymphoma that we consider to be derived from germinal center cells is centrocytic lymphoma. As shown in Fig. 6, it is composed only of cells that are similar to centrocytes. The number of SIg-positive cells and the SIg density were usually high. We found a nearly equal number of C3b and C3d receptors, a low percentage of mouse erythrocyte-binding cells, and a low T-cell content. This marker constellation is clearly different from that of B-CLL.

##### Centroblastic-Centrocytic Lymphoma

A schematic representation of the cytologic composition of the second type of germinal center cell lymphoma, centroblastic-centrocytic lymphoma, is shown in Fig. 7. Centroblastic-centrocytic lymphoma imitates follicular lymphoid hyperplasia in its cytology. Centroblasts and centrocytes proliferate side by side, but centrocytes predominate.

#### Immunologic Markers in the Different

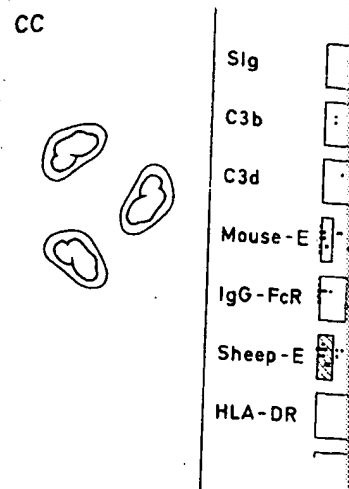


Fig. 6. Centrocytic lymphoma (CC). For

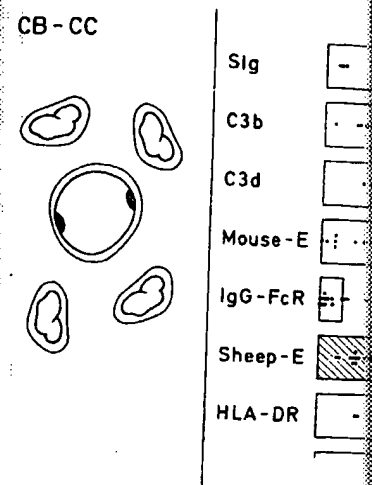
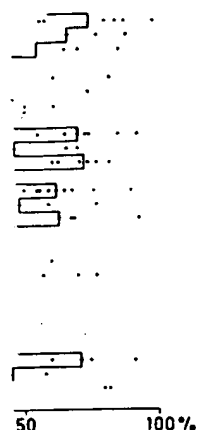
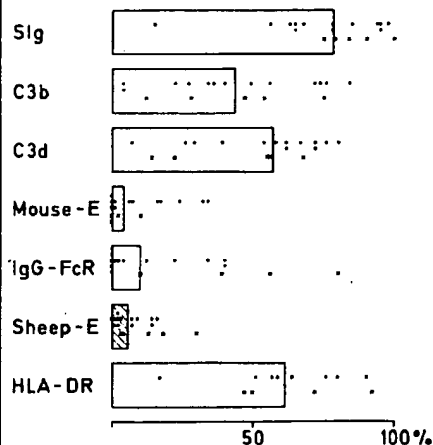


Fig. 7. Centroblastic-centrocytic lymphoma

In contrast to centrocytic lymphoma, the marker profile varied. The most important markers found to be C3 receptors, mouse erythrocyte receptors. The number of C3b receptor-positive cells. The percentage was relatively high (between that of B-CLL and T-cell content).



CC



**Fig. 6. Centrocytic lymphoma (CC).** For explanations see Fig. 1

nocytoma, LP-Ic). For explanations see Fig. 1

is similar to that of centroblastic-cen-

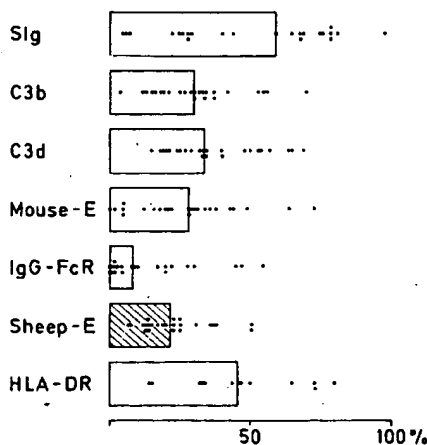
ined morphologically as a mixture of cytoid cells, interspersed with a large mass not similar to any other pattern.

### Cells

ter cell lymphoma.

As shown in Fig. 6, it is composed only of a number of SIg-positive cells and the SIg is equal number of C3b and C3d receptor-binding cells, and a low T-cell content. It is different from that of B-CLL.

composition of the second type of ger-  
-centrocytic lymphoma, is shown in  
a imitates follicular lymphoid hyper-  
-ocytes proliferate side by side, but cen-



**Fig. 7. Centroblastic-centrocytic lymphoma (CB-CC).** For explanations see Fig. 1

In contrast to centrocytic lymphoma, the number of SIg-positive tumor cells varied. The most important markers for centroblastic-centrocytic lymphoma were found to be C3 receptors, mouse erythrocyte receptors, and sheep erythrocyte receptors. The number of C3b receptor-positive cells was nearly equal to that of C3d receptor-positive cells. The percentage of mouse erythrocyte receptor-positive cells was relatively high (between that of B-CLL and centrocytic lymphoma). The same holds true for the T-cell content.

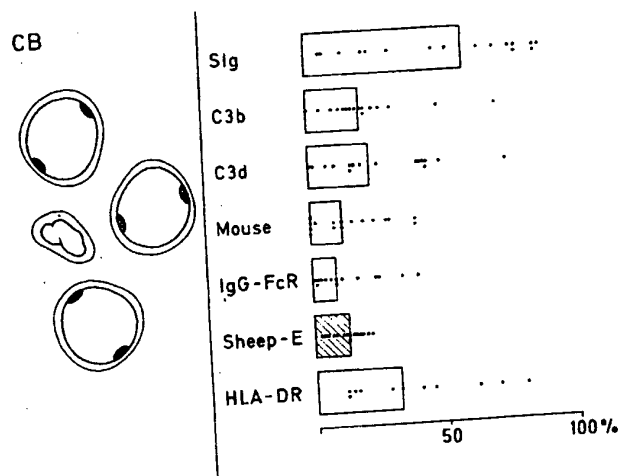


Fig. 8. Centroblastic lymphoma (CB). For explanations see Fig. 1

### Centroblastic Lymphoma

The third type of lymphoma that we consider to be derived from germinal center cells is characterized by a predominance of cells resembling centroblasts, as shown by Fig. 8. Accordingly, we chose the name centroblastic lymphoma.

Centroblastic lymphoma also showed the equal numbers of C3b and C3d receptors characteristic of germinal center cells. On the whole, the marker profile of centroblastic lymphoma resembled that of centroblastic-centrocytic lymphoma, although the median values were somewhat lower.

### Lymphoblastic Lymphomas

In this study we present immunologic and enzyme cytochemical data only on the two types of lymphoblastic lymphoma that can be classified by morphological criteria. These are lymphoblastic lymphoma of the Burkitt type and that of the convoluted-cell type.

### Lymphoblastic Lymphoma of the Burkitt Type

We analyzed the phenotype of five cases of lymphoblastic lymphoma of the Burkitt type. As Fig. 9 shows, the cells of all cases were characterized by the presence of a relatively large amount of SIg, but absence of C3 receptors and mouse erythrocyte receptors. IgG-Fc receptors were found in only one case. The T-cell content was consistently low.

Thus, lymphoblastic lymphoma of the Burkitt type is clearly different from all other types of lymphoma in its marker constellation. The difference between lymphoblastic lymphoma of the Burkitt type and germinal center cell lymphomas is especially important, because it does not agree with the concept that lymphoblastic

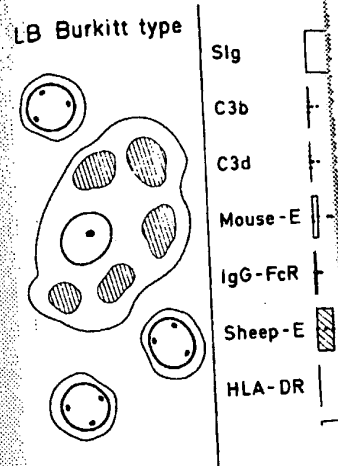


Fig. 9. Lymphoblastic lymphoma of Burkitt type.

lymphoma of the Burkitt type is has been proposed by several at

### Lymphoblastic Lymphoma of the Burkitt Type Including T-cell Acute Lymphoblastic Lymphoma

We have collected 31 cases of lymphoblastic lymphoma of the Burkitt type. In addition, human T-cell lymphoma was described recently (Stein et al. 1980). The first marker constellation was characterized as follows. C3 receptors and mouse erythrocyte receptors were consistently absent, and acid nonspecific esterase activity was found. This marker profile corresponds to the convoluted-cell type of lymphoma. For this reason, we designated this subtype of lymphoblastic lymphoma as the second marker constellation was characterized by the simultaneous presence of C3 receptors and the presence of acid phosphatase activity. This marker profile corresponds to the T-cell type of lymphoma, which develop during fetal life. In the T-cell type of lymphoma, we observed in one case. It was characterized by the presence of sheep erythrocyte receptors and the presence of sheep erythrocyte receptors at 37 °C. Acid phosphatase activity was absent or deficient. This constellation

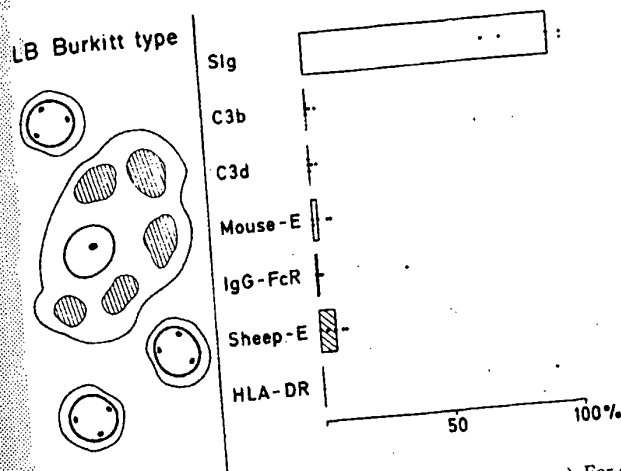


Fig. 9. Lymphoblastic lymphoma of Burkitt type (LB Burkitt type). For explanations see Fig. 1

der to be derived from germinal center cells resembling centroblasts, as shown in Fig. 1. The equal numbers of C3b and C3d receptors. On the whole, the marker profile of lymphoblastic-centrocytic lymphoma, although lower.

enzyme cytochemical data only on the basis that it can be classified by morphological features of the Burkitt type and that of the convoluted-cell type.

#### Type

Lymphoblastic lymphoma of the Burkitt type was characterized by the presence of C3 receptors and mouse erythrocyte receptors in only one case. The T-cell content

Burkitt type is clearly different from all other types. The difference between lymphoblastic and germinal center cell lymphomas is in agreement with the concept that lymphoblastic

#### Lymphoblastic Lymphoma of the Convoluted-cell Type, Including T-cell Acute Lymphoblastic Leukemia

We have collected 31 cases of lymphoblastic lymphoma of T-cell type. All of these cases were diagnosed with the aid of sheep erythrocyte receptors as T-cell marker. In addition, human T-cell lymphocyte antigen was used in some cases. As described recently (Stein et al. 1980), four main marker constellations could be distinguished. The first marker constellation was found in eight cases and can be characterized as follows. C3 receptors were present, or in some cases, lacking; sheep erythrocyte receptors were consistently lacking; strong, focal acid phosphatase activity was found, and acid nonspecific esterase activity was absent or deficient. This marker profile corresponds to that of the majority of fetal thymocytes of the 12th week of gestation. For this reason, we have used the designation "prethymocytic subtype of lymphoblastic lymphoma" for neoplasms with this type of pattern. The second marker constellation was found in 19 cases. It was characterized by the simultaneous presence of C3 receptors (both subtypes) and sheep erythrocyte receptors and the presence of acid phosphatase activity, but usually the absence of acid nonspecific esterase activity. This marker constellation is similar to that of prothymocytes, which develop during fetal life from prethymocytes, by acquiring sheep erythrocyte receptors. Thus, we have called this subtype of neoplasm the "prothymocytic subtype of lymphoblastic lymphoma." The third marker constellation was observed in one case. It was characterized by the complete absence of C3 receptors and the presence of sheep erythrocyte receptors that were capable of binding at 37 °C. Acid phosphatase activity was found, but nonspecific esterase activity was absent or deficient. This constellation fits that of postnatal thymocytes. Therefore,

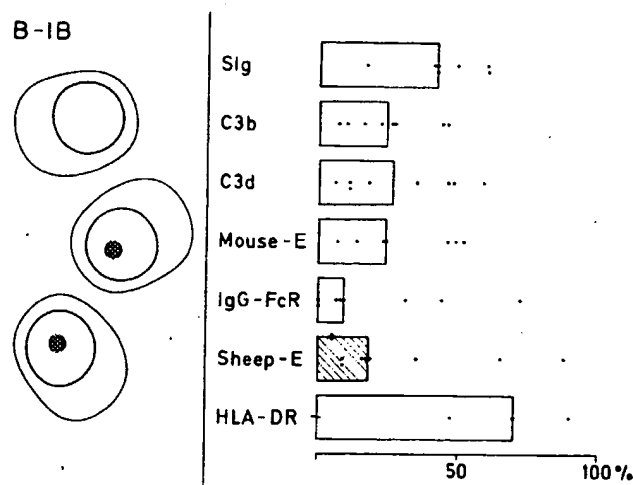


Fig. 10. Immunoblastic lymphoma of B-cell type (B-IB). For explanations see Fig. 1

the term "mature thymocytic subtype of lymphoblastic lymphoma" was chosen. The fourth marker constellation was found in three cases. It was characterized by the lack of C3 receptors, the presence of sheep erythrocyte receptors that were capable of binding only at 4 °C, and the presence of both acid phosphatase and non-specific esterase activity. This pattern corresponds to that of a majority of peripheral blood T cells.

#### Immunoblastic Lymphoma

In eight of nine cases of immunoblastic lymphoma we found SIg on the tumor cells (Fig. 10). We analyzed three cases for HLA-DR and found this antigen to be present on most tumor cells. It is interesting to note that in three cases the percentage of mouse erythrocyte rosette-forming cells exceeded 45%, and that in two cases the percentage of sheep erythrocyte rosette-forming cells exceeded 60%. The B-cell nature of these two cases could also be clearly demonstrated by monotypic light chain staining of the tumor cells.

Thus, all eight cases were B-cell-derived. Unfortunately, the results of the marker studies we performed do not permit us to distinguish between B-immunoblastic lymphoma and centroblastic lymphoma.

The cells of the ninth case lacked SIg and formed sheep erythrocyte rosettes. Thus, this case of immunoblastic lymphoma was identified as T-cell-derived.

#### Final Remarks

The immunologic data presented in this report are summarized in Table 3. We are aware of the problems connected with condensing data into this form. Nevertheless, some readers may find the table helpful, especially because we have limited the data to those on the types of NHL derived from B cells.

#### Immunologic Markers in the Differentiation

Table 3. Immunologic features of the B-cell

	CLL	PLL	HC
SIg			
C3bR			
C3dR			
Mouse ER			
IgG-FcR			
HLA-DR			
Sheep ER	+	+	+

I = 10%, II = 20%, III = 30% etc., + = ca.  
CLL = chronic lymphocytic leukemia of  
cell leukemia; LP-IC = lymphoplasmacytic  
lymphoma; CB-CC = centroblastic-ccc  
LB-BU = lymphoblastic lymphoma of the  
type

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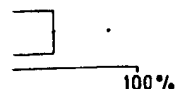
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kemic lymphocytes with special referen  
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production by tumor cells in non-Hodg

Table 3. Immunologic features of the B-cell-derived lymphomas of the Kiel classification

	CLL	PLL	HCL	LP-IC	CC	CB-CC	CB	LB-BU	IB
SIg									
C3bR									
C3dR									
Mouse ER			I				I		
IgG-FcR					I	I	I		I
HLA-DR									
Sheep ER	+	+	+	++	+	+++	++		+++

I = 10%, II = 20%, III = 30% etc., + = ca. 5%, ++ = ca. 10%, +++ = ca. 20%

CLL = chronic lymphocytic leukemia of B cell type; PLL = prolymphocytic leukemia; HCL = hairy cell leukemia; LP-IC = lymphoplasmacytic/cytoid lymphoma (all 3 subtypes); CC = centrocytic lymphoma; CB-CC = centroblastic-centrocytic lymphoma; CB = centroblastic lymphoma; LB-BU = lymphoblastic lymphoma of the Burkitt type; IB = immunoblastic lymphoma of the B cell type



3). For explanations see Fig. 1

mphoblastic lymphoma" was chosen in three cases. It was characterized by erythrocyte receptors that were capable of both acid phosphatase and non-phosphatase activity.

phoma we found SIg on the tumor cells. HLA-DR and found this antigen to be present in three cases the percentage of cells exceeded 45%, and that in two cases the percentage of cells exceeded 60%. The B-cell nature was demonstrated by monotypic light chain expression.

Unfortunately, the results of the immunohistochemical studies did not allow us to distinguish between B-immunoblastic lymphoma and T-cell lymphoma. In one case, the tumor cells formed sheep erythrocyte rosettes. In another case, the tumor cells were identified as T-cell-derived.

The immunologic features of the lymphomas are summarized in Table 3. We are presenting data into this form. Nevertheless, especially because we have limited data on the immunologic features derived from B cells.

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## Biochemical Analysis and Those of Lymphomas by High-Resolution

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**Summary.** The technique was applied to crude mesenchymal tissue samples, and those of lymphomas of different typology, tissue specificity, and prevalence of the latter aspects. The technique, however, is not a substitute for the more extensive, but more objective, comparison of objective, comparative maps is described.

**Key words:** Protein map, characterization - Silver

## Introduction

The classification of tumors is supplemented by a relative assessment of the presence or absence of the F<sub>c</sub>-fragment of heavy chain, and certain specific (isoelectric identity) and *does* (its function) (expressed gene products) is determined by biochemical parameters which are readily available. The current

\* Supported by grants of the Deutsche Forschungsgemeinschaft, University of Kiel.

Offprint requests to: K. Westerbrink.

Abbreviations: IEF = isoelectric focusing; SDS = sodium dodecylsulphate; C = centrocytoma; CBCC = centroblastoma; plasmacytoma



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## TRANSCRIPTIONAL REGULATION OF THE HUMAN IgE RECEPTOR (FcεRII/CD23) BY EBV

### Identification of EBV-Responsive Regulatory Elements in Intron 1<sup>1</sup>

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EBV infection of B cells induces the B cell activation Ag, CD23 (FcεRII). CD23 remains constitutively expressed at high levels in all EBV-immortalized B cells and likely plays an important role in the initiation and maintenance of immortalization by EBV. By utilizing an EBV-negative Burkitt's lymphoma line (BJAB) and EBV-positive sublines derived from it by *in vitro* infection, we have examined the molecular mechanisms involved in the regulation of CD23 by EBV. By nuclear runoff analysis, we have found that induction of CD23 is mediated by transcriptional activation that occurs in the presence of the transformation-competent B958 virus but not in the presence of the nontransforming P3HR-1 strain of EBV. To identify EBV-responsive transcriptional regulatory elements of CD23, we have performed reporter gene assays using plasmids containing fragments of the CD23 gene derived from its 5' terminus and adjacent flanking region transfected into EBV-positive and -negative BJAB lines. We have identified a 534-bp fragment of the gene which enhances transcription from a heterologous promoter (SV40) and reporter gene (chloramphenicol acetyltransferase) only in the presence of transformation-competent strains of EBV. Deletion of 144 bp of intron 1 from the 3' end of this fragment results in loss of EBV-responsive enhancer activity. The finding of an EBV-responsive enhancer element of CD23 is supported by mobility shift assays that demonstrated the formation of specific DNA-protein complexes between nuclear protein from transforming EBV-positive cells and the 144-bp intron sequence. These studies suggest that the transcriptional activation of CD23 by transforming strains of EBV involves regulatory elements that are located within the first intron of the gene.

EBV, a B lymphotropic human herpesvirus, is causally associated with several human B cell malignancies, including endemic Burkitt's lymphoma and B cell lymphomas in immunocompromised hosts (1, 2). *In vitro*, EBV

transforms normal B cells to immortalized lymphoblastoid lines capable of continuous proliferation (3). Because the immortalization of B cells *in vivo* may represent an early step in the genesis of the EBV-associated malignancies, an elucidation of the molecular events involved in immortalization may be important in understanding EBV-associated lymphomagenesis. Although the molecular events that initiate and maintain the immortalization of B cells by EBV have not yet been fully elucidated, *in vitro* studies suggest that the process may involve the B cell activation pathway and autocrine stimulation. Thus, EBV-infected B cells express B cell activation Ag (e.g., CD23, CD30, CD39, CD40, and CD44) and cellular adhesion molecules (e.g., ICAM-1, LFA-1, and LFA-3) that are expressed on mitogen-stimulated B cells (4-10). In addition, EBV-immortalized lymphoblastoid lines secrete factors with B cell growth factor activity, and some of these factors stimulate autocrine growth of serum-deprived lymphoblastoid lines (11-15).

The B cell activation Ag, CD23, also known as the low affinity IgE receptor (FcεRII) (16-18), may play a central role in immortalization of EBV-infected B cells. CD23 is rapidly induced by EBV and expressed at high levels in all immortalized B cells (6, 16), and soluble subfragments of CD23 (sCD23) stimulate autocrine growth of lymphoblastoid lines (14). Furthermore, the expression of CD23 appears to be closely associated with the immortalizing functions of EBV because the nontransforming P3HR-1 strain of EBV does not induce CD23 (7), and immortalized cells arise only from EBV-infected cells that express CD23 (19). Recently, two species of CD23, types a and b, which differ by six amino acids in the cytoplasmic amino terminus and utilize different promoters and 5' exons have been identified (20). Type a expression is restricted to B cells whereas type b is expressed in other hematopoietic cells and is inducible by IL-4 (20). Although type b CD23 is not found in unstimulated B cells, both type a and type b species of CD23 are expressed in EBV-immortalized B cells (20, 21).

The molecular mechanisms involved in the regulation of CD23 by EBV remain uncertain. Indeed, it is not known whether CD23 is induced by transcriptional activation or post-transcriptional events. Although the viral proteins EBNA-2 and LMP-1 have been implicated in the regulation of CD23 (21-25), the mechanism by which these proteins mediate their effects on CD23 expression is unclear. The goals of this study are to determine whether induction of CD23 by EBV involves a transcriptional mechanism and to identify the transcriptional reg-

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By utilizing the EBV-negative and -positive BJAB lines as well as additional nonisogenic EBV-negative and -positive lines we have found that induction of CD23 in EBV-positive lines is mediated in part by transcriptional activation and involves EBV-responsive transcriptional regulatory elements that specifically bind nuclear protein(s) present only in EBV-positive lines.

**Cell lines.** BJAB cells were derived from a human EBV-negative Burkitt-like lymphoma (26, 27). BJAB-B1 and BJAB-B958 are EBV-positive sublines of BJAB which were derived from BJAB cells by in vivo infection with the P3HR-1 and B958 strains of EBV, respectively (28). BJAB-B1 has been clonally derived from P3HR-1-infected BJAB cells (33). BJAB-B1-107 was subcloned from the BJAB-B1 line and is an EBV-negative revertant line that has lost the EBV genome (34). The LB-11-23 and LB-X50-7 lines are EBV-positive immortalized lymphoblastoid lines that were derived by infection of umbilical cord lymphocytes with FF-41 and B958 strains of EBV, respectively. LB-11-23 was derived in this laboratory; LB-X50-7 was generously provided by G. Miller (Yale University). Louckes was derived from an EBV-negative Burkitt's lymphoma and was provided by M. Lewis (Bios, Inc.). All lines were maintained in RPMI 1640 plus 10% FBS plus penicillin and streptomycin. The presence or absence of EBV DNA and viral nuclear Ag in all lines was confirmed by nucleic acid hybridization studies and anti-complement immunofluorescence studies, respectively (29, 30). The origin and characterization of these cell lines are summarized in Table I.

**Nuclear runoff transcription assay.** Runoff transcription assays were performed essentially as described previously (30) using a modification of the method described by Lintal et al. (35). Cells were harvested in exponential phase of growth for all runoff transcription assays. An equivalent amount of radiolabeled RNA (10<sup>7</sup> cpm) from each cell line was hybridized to denatured cDNA probes (1 µg) in 2.5 ml of hybridization solution. The amount of radiolabeled RNA hy-

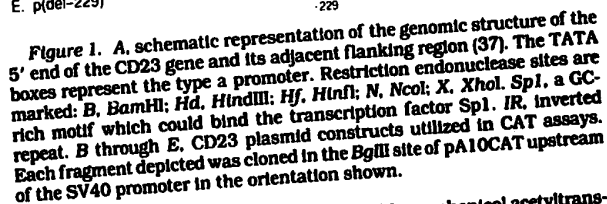
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Cell Line	Origin	Superinfecting Strain of EBV	EBNA <sup>a</sup>	EBV DNA <sup>b</sup>
BJAB	Lymphoma	Absent	Absent	Absent
BJAB-B958	BJAB	B958	Present	Present
BJAB-B1	BJAB	P3HR-1	Present	Present
BJAB-B1-107	BJAB-B1	Absent	Absent	Absent
LB-11-23	Lymphocytes	FF41	Present	Present
LB-X50-7	Lymphocytes	B958	Present	Present
Louckes	Lymphoma	Absent	Absent	Absent

<sup>b</sup> The presence or absence of EBV DNA was determined by Southern blot analysis using whole EBV genome as a probe (29).

**Construction of plasmids for CAT<sup>a</sup> assays.** A series of plasmid constructs was generated by cloning fragments of a genomic clone of the 5' region and adjacent flanking sequence of CD23 into the pA10CAT plasmid. The pA10CAT plasmid contains the bacterial CAT gene driven by the SV40 21-bp repeated promoter element and early gene TATA box and followed by an SV40 t intron and poly(A) addition site (36). The genomic clone of CD23 was generously provided by Hans Hofstetter (37). The 5' terminus and flanking region of CD23 have been previously sequenced and characterized; consensus sequences that may represent putative regulatory elements have been identified (Fig. 1A) (37). Fragments of varying length as depicted in Figure 1 were derived from this clone by restriction endonuclease digestion and gel purification and cloned by blunt end or cohesive end ligation into the *Eg*III site of pA10CAT just upstream of the SV40 promoter. Plasmid constructs containing each fragment of CD23 depicted in Figure 1 cloned in the 5' to 3' direction relative to the SV40 promoter were utilized in the CAT assays.

**Mobility shift assay.** Nuclear extracts from each cell line were prepared essentially as described by Dignam et al. (41), and mobility shift assays were performed as described by Hennighausen and Lubon (42). In brief, DNA fragments were radiolabeled at their



<sup>3</sup> Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; EBNA, Epstein-Barr virus nuclear Ag; FcγRII, lymphocyte IgG receptor; LMP, latent membrane protein; TBS, Tris-buffered saline.

termini by a fill-in reaction with  $^{32}$ P-deoxynucleotides to a sp. act. of  $10^4$  to  $10^5$  cpm/ng. For each mobility shift assay, 1  $\mu$ g of extract was preincubated in a 25- $\mu$ l reaction with varying amounts of poly(dI-dC) in 10 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, 75 mM KCl, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100, and 12.5% glycerol for 30 min at room temperature. After the addition of 1 ng of radiolabeled DNA fragment, extracts were incubated for an additional 30 min and then resolved by electrophoresis through a 5% acrylamide gel in a running buffer of 45 mM Tris, 45 mM boric acid, 4 mM EDTA at 25 mA for 3 h. Gels were dried before autoradiography. In preliminary experiments it was determined that maximum resolution of DNA-protein complexes occurred using 2 to 8  $\mu$ g of poly(dI-dC) in each reaction. For competition experiments, unlabeled competitor DNA in increasing concentrations was included in the preincubation reaction with a defined amount of poly(dI-dC) (4 or 8  $\mu$ g). Double-stranded oligonucleotides of the Sp1, Ap1, and Ap2 consensus sequences were purchased from Stratagene (La Jolla, CA).

## RESULTS

**RNA blot analysis of CD23 expression.** To determine whether EBV infection of the EBV-negative BJAB line is associated with induction of CD23 expression, RNA blot analysis was performed on the B958- and P3HR-1-converted BJAB lines using a cDNA probe that detects both type a and type b species (43) (Fig. 2). CD23 transcripts were undetectable in both EBV-negative BJAB lines, BJAB and BJAB-B1-107, and in the P3HR-1-converted BJAB line, BJAB-B1. In contrast, abundant amounts of CD23 mRNA were detectable in the B958-converted BJAB line, BJAB-B958. Examination of the nonisogenic lines revealed large amounts of CD23 mRNA in both EBV-positive lymphoblastoid lines, LB-11-23 and LB-X50-7, and no detectable transcripts in the EBV-negative Louckes line. These studies demonstrated that conversion of the BJAB line with the transforming B958 strain of EBV is associated with induction of CD23 whereas the nontransforming P3HR-1 strain does not induce CD23 mRNA expression in BJAB cells.

**Nuclear runoff analysis of CD23 transcription.** The molecular mechanism by which transforming strains of EBV induce CD23 mRNA has not been elucidated previously. To determine whether increased levels of CD23 transcripts in B958-positive BJAB cells result from transcriptional activation or post-transcriptional events, nuclear runoff analysis was performed to determine the overall rates of transcription of CD23 in the EBV-positive and -negative BJAB lines. All cell lines were examined in at least two separate experiments using a near full length cDNA probe of CD23 which contains sequence shared by type a and type b species (43). The cDNA probes of  $\gamma$ -actin and HLA class I (b locus) plus plasmid DNA were

used as c ntr ls. Equal amounts of radiolabeled RNA from each cell line were hybridized to the blots. Hybridization to plasmid DNA was not detectable in these experiments.

The nuclear runoff assays showed that conversion of BJAB by the transforming B958 strain of EBV was associated with a 10- to 20-fold increase in the overall rate of transcription of CD23 relative to  $\gamma$ -actin or HLA (Fig. 3). The rate of transcription of CD23 in the P3HR-1-converted BJAB line and its EBV-negative revertant transformed BJAB line and its EBV-negative revertant was similar to the rate observed in BJAB cells (data not shown), demonstrating that P3HR-1 conversion of BJAB does not induce CD23 transcription. Nuclear runoff analysis of the nonisogenic lines, EBV-positive LB-X50-7 and EBV-negative Louckes, also revealed a 10- to 20-fold increase in the rate of transcription of CD23 relative to  $\gamma$ -actin and HLA in LB-X50-7 compared with Louckes cells. These studies indicate that induction of CD23 mRNA expression by the B958 strain of EBV results in part or entirely from transcriptional activation.

**Identification of EBV-responsive CD23 regulatory elements by CAT assay.** The transcriptional activation of CD23 by B958 virus in BJAB cells suggests that there may be transcriptional regulatory elements of CD23 which are responsive to EBV. To identify EBV-responsive regulatory elements within or adjacent to CD23, a series of plasmid constructs containing DNA fragments of varying length from the 5' and flanking region of CD23 cloned upstream of the CAT reporter gene driven by the SV40 promoter was utilized for transient transfections to identify functional transcriptional regulatory elements that enhance CAT expression in the presence of EBV. For each transfection experiment, the plasmid construct containing CD23 DNA was transfected in parallel with the pA10CAT plasmid for each cell line; the CAT activity of the test plasmid in a cell line was expressed relative to the CAT activity of the pA10CAT plasmid in that line after normalization of CAT activity to  $\beta$ -galactosidase activity in each extract. Each plasmid construct was tested a minimum of three times in most cell lines.

In the initial experiments there was significant enhancement of CAT activity with the p(-1330) and p(-229) constructs in the EBV-positive BJAB-B958, LB-X50-7, and LB-11-23 lines (Tables II and III). In contrast, there was no enhancement of CAT activity with these constructs in the three EBV-negative lines, BJAB, BJAB-B1-107, and Louckes and in the P3HR-1-converted BJAB line, BJAB-B1 (Tables II and III). These studies suggest that there may be an EBV-responsive enhancer element within a 534-bp fragment of CD23 which stretches from -229 bp into the first intron at +305 bp. This enhancer

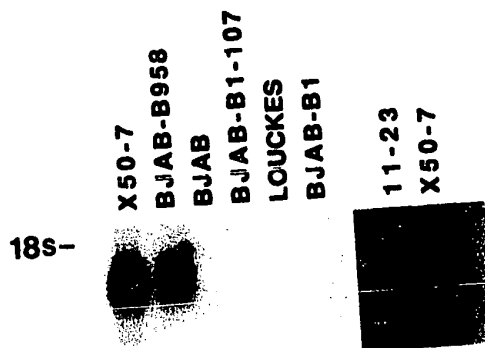


Figure 2. RNA blot analysis of CD23 transcripts in EBV-positive and -negative lines. Four  $\mu$ g of poly(A) RNA from LB-11-23 and LB-X50-7 and 10  $\mu$ g of poly(A) RNA from each other line were loaded on the gel.

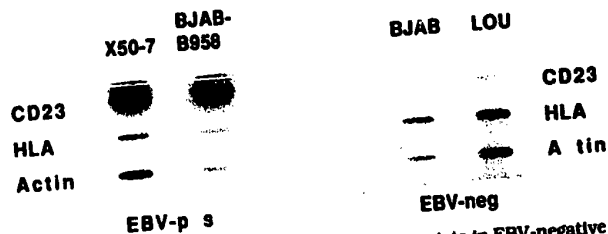


Figure 3. Nuclear runoff assays of CD23 transcripts in EBV-negative (BJAB and Louckes) and EBV-positive (BJAB-B958 and X50-7) cell lines. Nuclei were harvested from cells in the exponential phase of growth ( $2 \times 10^6$  cells/ml).  $10^7$  cpm of radiolabeled nascent transcripts from each line in 2.5 ml of hybridization solution were used for each blot.

TABLE II  
Effect of EBV on CAT reporter plasmids containing CD23 genomic fragments in BJAB-derived cell lines

Plasmid Construct	Relative CAT Activity <sup>a</sup>			
	EBV-Negative BJAB	EBV-Positive BJAB-B958	EBV-Positive BJAB-B1	EBV-Negative BJAB-B1-107
p(-1330)	0.79 (0.08) n = 3	5.7 (0.66) n = 4	0.89 (0.08) n = 3	0.78 (0.09) n = 2
p(-229)	0.74 (0.07) n = 3	6.1 (0.37) n = 5	0.93 (0.08) n = 4	0.92 (0.09) n = 2
p(+161)	0.84 (0.07) n = 3	3.4 (0.29) n = 4	0.96 (0.07) n = 3	0.84 (0.08) n = 2
p(del-229)	0.80 (0.08) n = 4	1.4 (0.10) n = 5	0.79 (0.09) n = 3	0.98 (0.09) n = 2

<sup>a</sup> Expressed as CAT activity (percent acetylation of chloramphenicol) of the plasmid construct relative to the CAT activity of the control pA10CAT plasmid after normalization of CAT activity to  $\beta$ -galactosidase activity. The value shown is the mean from several (n) transfections; the SE is indicated in parentheses.

TABLE III  
Effect of EBV on CAT reporter plasmids containing CD23 genomic fragments in nontsogenic cell lines

Plasmid Construct	Relative CAT Activity <sup>a</sup>		
	EBV-Negative Louckes	EBV-Positive LB-X50-7	EBV-Positive LB-11-23
p(-1330)	0.72 (0.03) n = 3	6.1 (0.84) n = 5	7.1 (0.94) n = 3
p(-229)	0.75 (0.05) n = 3	6.9 (0.74) n = 5	8.9 (0.71) n = 3
p(+161)	0.79 (0.07) n = 2	3.2 (0.67) n = 4	3.8 (0.72) n = 4
p(del-229)	0.75 (0.09) n = 2	1.3 (0.16) n = 5	1.0 (0.15) n = 3

<sup>a</sup> Expressed as CAT activity (percent acetylation of chloramphenicol) of the plasmid construct relative to the CAT activity of the control pA10CAT plasmid after normalization of CAT activity to  $\beta$ -galactosidase activity. The value shown is the mean from several (n) transfections; the SE is indicated in parentheses.

element is not functional in the presence of the nontransforming P3HR-1 viral strain, however.

To localize the EBV-responsive enhancer element(s) within the 534-bp fragment, additional CAT assays were performed using two plasmid constructs with subfragments of the 534-bp fragment, p(+161) which contains 144 bp of sequence from intron 1 and p(del-229) which contains exon 1 and the promoter of type A CD23 plus 5' adjacent flanking sequence (Fig. 1). Using the p(+161) plasmid, some enhancement of CAT activity was retained in the 144-bp intron sequence of CD23 in all three EBV-positive lines infected with transforming strains of EBV (Tables II and III). However, the enhancer activity of this fragment was substantially reduced compared with the enhancement observed with the larger 534-bp fragment. In the EBV-negative lines and the P3HR-1-converted BJAB line, there was no enhancement of CAT activity with this 144-bp fragment. Using the p(del-229) plasmid, there was minimal enhancement of CAT activity in EBV-positive lines and no enhancement in EBV-negative lines (Tables II and III). These experiments suggest that there is an EBV-responsive transcriptional regulatory element in the first intron of CD23. However, additional regulatory elements must be present in the 5' region adjacent to this fragment because the 534-bp fragment that contains the intron sequence, plus the first exon, type A promoter, and adjacent 5'-flanking region demonstrated substantially more CAT activity in the EBV-positive lines than did the 144-bp fragment derived from the first intron.

**Mobility shift assays.** The reporter gene assays suggested that there may be functional EBV-responsive enhancer activity in a 144-bp fragment derived from the first intron of CD23. To determine if there are nuclear proteins that specifically bind to this fragment, mobility shift assays were performed using the 144-bp fragment and nuclear extracts derived from both EBV-positive and -negative cell lines (Fig. 4, A and B). These experiments

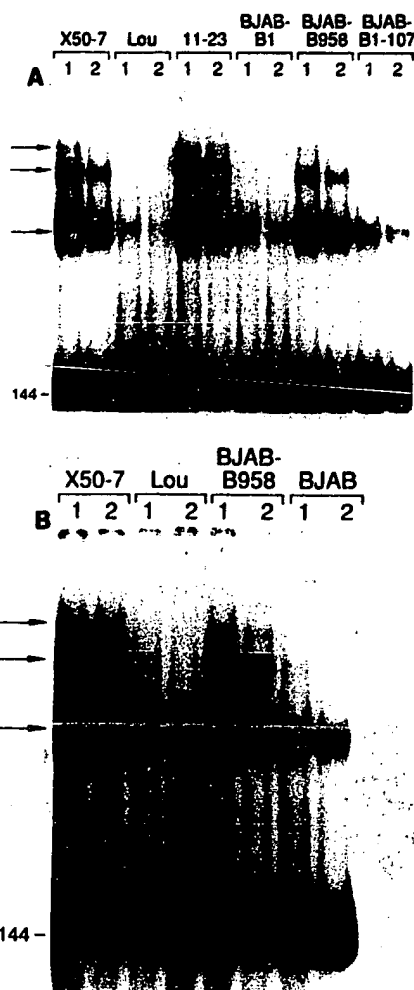


Figure 4. Mobility shift assays using the 144-bp fragment of CD23 (see Fig. 1D) and nuclear extracts from the three EBV-positive lines infected with transforming virus (X50-7, 11-23, and BJAB-B958), the EBV-positive line infected with the nontransforming strain of EBV (BJAB-B1), and the three EBV-negative lines (BJAB, BJAB-B1-107, and Louckes). Lanes 1 and 2 show extracts incubated with 4 and 8  $\mu$ g of poly(dI-dC). The arrows indicate the three shifted bands representing DNA-protein complexes that are absent or less abundant in the EBV-negative and P3HR1-infected lines compared with the EBV-positive lines. Different extract preparations were used for each gel.

revealed three bands of retarded mobility in the three EBV-positive lines, BJAB-B958, LB-X50-7, and LB-11-23. In contrast, only one of these three bands was observed consistently in the EBV-negative lines and the P3HR-1-converted BJAB line, and this band was markedly reduced in intensity relative to the EBV-positive lines. These findings were confirmed with at least two different preparations of extracts, indicating that the observed differences between the EBV-positive and -neg-

active lines did not simply reflect variability in extract preparation. These studies suggest the presence of nuclear protein(s) in the three EBV-positive lines that interact with sequence in the 144-bp fragment; these proteins are substantially less abundant or absent in all three EBV-negative lines examined.

To determine the specificity of the DNA-protein interactions observed, competition assays were performed using unlabeled 144-bp fragment or heterologous DNA (plasmid DNA of 150 bp in length) in increasing concentrations (Fig. 5). Preincubation of the EBV-positive nuclear extracts with excess unlabeled specific probe reduced the formation of the DNA-protein complexes to a greater extent than an equimolar amount of heterologous unlabeled probe. Similar results were obtained using different heterologous DNAs as nonspecific competitor. These findings suggest that these bands may represent specific DNA-protein interactions.

Previous sequence analysis of the 5' region of CD23 revealed several potential regulatory elements, including a GC-rich motif, 5'-GGGGGCGGGG-3', which could bind the transcription factor Sp1, at the beginning of the first intron of the type A CD23 (Fig. 1) (37). To determine whether the DNA-protein interactions observed using the 144-bp fragment involve the Sp1-binding site, mobility shift assays were performed using increasing amounts of a 22-bp double-stranded oligonucleotide containing the sequence of the Sp1 binding site (Fig. 6). These experiments showed that there was no reduction in the formation of the DNA-protein complexes using the 144-bp fragment and nuclear extracts from the EBV-positive lines LB-X50-7 (Fig. 6), LB-11-23, and BJAB-B958 (not shown) in the presence of up to 200-fold molar excess of Sp1 oligonucleotide. Similar results were obtained using double-stranded oligonucleotides containing the Ap1 and Ap2 binding sites. These studies suggest that the observed DNA-protein complexes formed in the presence of

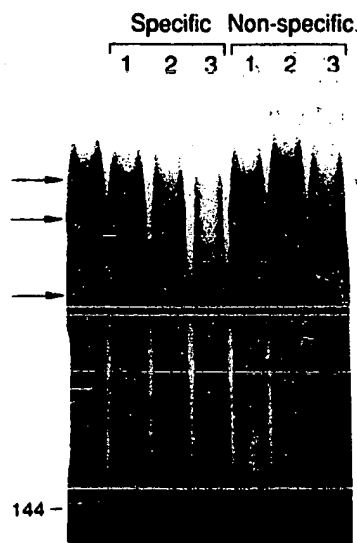


Figure 5. Mobility shift assay using the 144-bp fragment of CD23 (see Fig. 1D) and nuclear extract from LB-X50-7 cells in the presence of increasing amounts of unlabeled specific 144-bp probe or nonspecific heterologous DNA of the same size derived from plasmid DNA. Reactions were incubated in the presence of 4  $\mu$ g of poly(dI-dC). Lane 0, no competitor DNA; lanes 1, 2, and 3 show extracts incubated with 10-, 50-, and 100-fold molar excess of unlabeled DNA, respectively. The arrows indicate the three DNA-protein complexes that are absent or less abundant in EBV-negative and P3HR1-infected lines compared with EBV-positive lines.

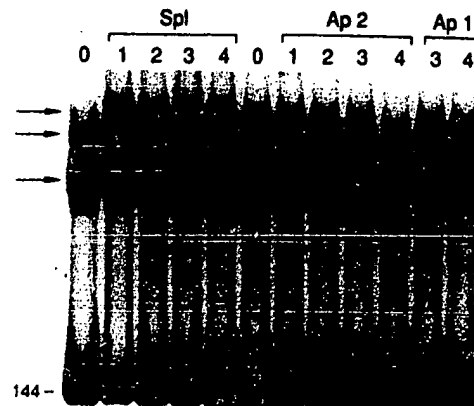


Figure 6. Mobility shift assay using the 144-bp fragment of CD23 (see Fig. 1D) and nuclear extracts from LB-X50-7 cells in the presence of increasing amounts of unlabeled double-stranded oligonucleotides containing the Sp1, Ap1, or Ap2 binding sequence. Reactions were incubated in the presence of 4  $\mu$ g of poly(dI-dC). Lane 0, no oligonucleotide. Lanes 1, 2, 3, and 4 show reactions incubated with 10-, 50-, 100-, and 200-fold molar excess of oligonucleotide, respectively. The arrows indicate the three DNA-protein complexes that are absent or less abundant in EBV-negative and P3HR1-infected lines compared with EBV-positive lines.

the 144-bp fragment and nuclear extracts from the EBV-positive lines do not involve the Sp1 binding site. Because there are no other consensus sequences of known enhancer elements within the 144-bp fragment, it is likely that the observed DNA-protein complexes involve a novel EBV-responsive transcriptional regulatory element.

#### DISCUSSION

These studies extend our understanding of the molecular mechanisms involved in the regulation of CD23 expression by EBV in human B cells. By utilizing a model system of isogenic EBV-positive and -negative B cell lymphoma lines, we have demonstrated that induction of CD23 mRNA expression by EBV clearly results from transcriptional activation. Although our studies do not exclude the possibility that post-transcriptional regulation may be involved as well, it is clear that increased transcription of CD23 is an important mechanism by which EBV activates CD23. We have also shown that transformation-competent virus is necessary to activate transcription of CD23 because the nontransforming P3HR-1 strain of EBV failed to increase transcription of CD23 in BJAB cells.

The transcriptional activation of CD23 by EBV suggests that there may be transcriptional regulatory elements of CD23 which are responsive to the presence of transforming EBV. In support of this hypothesis, we have identified a 534-bp fragment of CD23 (-229 to +305 bp) which contains functional enhancer activity in reporter gene assays in the presence of the transforming B958 or FF41 strains of EBV. This fragment does not enhance transcription in the absence of EBV or in the presence of the nontransforming P3HR-1 strain of EBV. This latter observation is consistent with the inability of P3HR-1 virus to induce CD23 expression and indicates that P3HR-1 virus does not express the requisite viral products necessary for transcriptional activation of CD23 via EBV-responsive enhancer element(s) of the gene that we have identified. Previous studies using gene transfer techniques have strongly implicated both EBNA-2 and LMP-1 in the induction of CD23 (21-25). Because P3HR-1 virus is deleted in the region of the genome that encodes

EBNA-2 (1), our studies using whole virus-infected cells are consistent with the concept that EBNA-2 may be involved in the transcriptional activation of CD23.

The EBV-responsive enhancer activity that we have identified was maximal in the 534-bp fragment that includes approximately 200 bp of adjacent 5'-flanking sequence of CD23 and extends into intron 1 of the type a CD23. Interestingly, neither subfragment of this 534-bp piece retained maximum EBV-responsive enhancer activity; the 5' end of the fragment (-229 to +161 bp) demonstrated minimal enhancer activity in EBV-positive lines whereas the 3' end of the fragment (+161 to +305 bp) retained only partial EBV-responsive enhancer function. These observations suggest that there may be more than one enhancer element within the 534-bp fragment which function cooperatively to enhance CD23 transcription in the presence of EBV. Alternatively, the enhancer element may lie adjacent to or span the cleavage site that was utilized in generating the two subfragments, resulting in some loss of function in the 3' subfragment of the 534-bp piece.

The finding of a potential EBV-responsive enhancer element of CD23 by functional studies is strongly corroborated by the mobility shift assays that demonstrate specific DNA-protein complexes between the 144-bp fragment from intron 1 and nuclear protein from cells infected with transforming strains of EBV. These DNA-protein complexes were not present or were substantially reduced when extracts from EBV-negative lines or the P3HR-1-converted BJAB line were examined. Our observations suggest that there are nuclear proteins present in EBV-transformed cells which specifically interact with the putative EBV-responsive enhancer element(s) that has been identified by functional studies. The nature and origin of these proteins remain unknown. Although they may represent viral products that directly trans-activate CD23, it is also possible that they represent cellular intermediate proteins that are activated by EBV infection.

Utilizing a different approach, Wang et al. (44) recently described a transcriptional regulatory element of CD23 which is responsive to EBNA-2. Using EBNA-2-transfected cells rather than whole virus-infected cells, Wang et al. identified an 800-bp fragment (-335 to +465) that enhanced reporter gene activity in the presence of EBNA-2. Interestingly, deletions from either the 5' or 3' end of this fragment resulted in some reduction in its EBNA-2-responsive enhancer activity, suggesting that there may be a cooperative effect between EBNA-2-responsive regulatory elements found in sequences flanking both sides of the type a promoter. Our findings using whole virus-infected cells likewise support the existence of cooperative EBV-responsive regulatory elements that flank the type a promoter of CD23.

The regulation of CD23 is complex in that the two distinct species of CD23, type a and type b, are generated by utilizing different promoters and alternative 5' exons, and these species are tissue specific in their expression (20). Type a CD23 is restricted to B cells and is constitutively expressed whereas type b CD23 is expressed in other cell types and is induced in IL-4-stimulated B cells (20). EBV-infected B cells express both types of CD23 mRNA (20, 21, 23), and by gene transfer techniques, it has been reported that EBNA-2 induces predominantly

type a whereas LMP induces predominantly type b species (21, 23). It is not known whether the type a and type b promoters utilize common transcriptional regulatory elements and trans-activating proteins or, alternatively, whether they are under the control of different transcriptional regulatory elements. Interestingly, Suter et al. (45) reported an IL-4-responsive regulatory element in the 250-bp fragment in the adjacent 5' region of the type a promoter. However, Suter et al. did not examine the first intron of CD23 for the presence of additional IL-4-responsive elements. Further studies of IL-4-stimulated B cells and other cell types, utilizing the reporter gene constructs described here as well as mobility shift assays with the 144-bp fragment of CD23, should permit elucidation of the transcriptional regulatory mechanisms involved in the generation of these mRNA species.

Our studies suggest that there is an EBV-responsive enhancer element that resides within a 144-bp sequence within the first intron of type a CD23. Although the transcriptional regulatory elements of many genes reside within adjacent 5'-flanking sequences, the localization of enhancers within introns has been well described for several genes, including the immunoglobulin H chain gene (38, 46) and the human  $\alpha 1(I)$  collagen gene (47). Because the enhancer activity we observed within the first intron is relatively weak, there may be cooperative effects between the intron enhancer element and a regulatory element in the adjacent 5'-flanking region. In addition, these studies do not eliminate the possibility that additional regulatory elements reside in other introns or in adjacent 3'-flanking sequences.

In conclusion, we have shown that induction of CD23 by transformation-competent EBV is mediated in part if not entirely by transcriptional activation. A 534-bp EBV-responsive enhancer element has been identified in the 5' region of the gene, and this regulatory element requires the presence of 144 bp of sequence from the first intron of the gene to retain maximum EBV responsiveness. The 144-bp sequence from intron 1 binds nuclear protein from EBV-positive cells, suggesting that it contains a regulatory element that interacts with trans-activating proteins present in virus-infected cells. The identification of the specific proteins and sequence involved in the regulation of CD23 by EBV remains to be elucidated.

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## EXPRESSION OF HUMAN LYMPHOCYTE IgE RECEPTOR (Fc $\epsilon$ RII/CD23) Identification of the Fc $\epsilon$ RII<sub>a</sub> Promoter and Its Functional Analysis in B Lymphocytes

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Two species, Fc $\epsilon$ RII<sub>a</sub> and Fc $\epsilon$ RII<sub>b</sub>, of the human low-affinity receptor for IgE (Fc $\epsilon$ RII/CD23) have recently been described. They differ by only six amino acids in the cytoplasmic N-terminus and are generated by different cell-specific transcriptional start sites that lead to distinct 5'-leader sequences in the corresponding mRNA. In this study, we present the analysis of the promoter which is regulating the expression of the B cell-specific Fc $\epsilon$ RII<sub>a</sub>. Our data show that this promoter is flanked by several long repetitive elements that are influencing transcription in the Burkitt lymphoma B cell line Jijoye. Serial deletions of the 5'-flanking region of the promoter revealed two major regulatory segments that have either inhibitory or enhancing effects on transcription. In addition, IL-4 caused a two- to four-fold up-regulation of the Fc $\epsilon$ RII<sub>a</sub> promoter activity and the DNA element responsible was mapped within the first 250 bp of the 5'-flanking region. These results were confirmed by transferring the DNA segment containing the putative IL-4 responsive element to a heterologous thymidine kinase promoter. It was concluded that IL-4 augments the Fc $\epsilon$ RII<sub>a</sub> expression by transcriptional regulation.

Low-affinity Fc $\epsilon$ RII<sup>3</sup> are found on the cell surface of a variety of hematopoietic cells including lymphocytes, monocytes, eosinophils, and platelets (1-5). The extracellular portion of Fc $\epsilon$ RII is cleaved proteolytically (6) and fragments are released which retain their ability to bind IgE (IgE-BF) (7-9). Several functions have been described for the membrane-bound Fc $\epsilon$ RII as well as for soluble IgE-BF. First, a role in IgE-mediated immunity, such as allergy and parasitic infections, was proposed. The Fc $\epsilon$ RII is involved in the IgE-mediated killing of schistosomes (10-12) and IgE-BF have been shown to regulate IgE synthesis *in vitro* (7, 13-15). Second, a function in B cell growth or differentiation was suggested. The expression

of Fc $\epsilon$ RII on B lymphocytes is developmentally restricted to mature  $\mu^+/\delta^+$  cells (16) and IgE-BF were shown to be growth factors for preactivated B cells (17). In addition, a yet unidentified B cell stimulatory factor and IgE are coordinately regulating B lymphocytic growth mediated by the Fc $\epsilon$ RII (18). Such multiple functions require a tight regulation, and indeed several factors have been described which influence the expression of the Fc $\epsilon$ RII. EBNA-2, an EBV-encoded protein, was shown to induce selectively the expression of the Fc $\epsilon$ RII on B cells (19). Furthermore, IL-4 acts as a strong inducer of the Fc $\epsilon$ RII on B cells (16, 20, 21) and monocytes (22), whereas IFN- $\gamma$  was found to reverse the enhancing effect of IL-4 on B lymphocytes (23).

Recently two different species of Fc $\epsilon$ RII (Fc $\epsilon$ RII<sub>a</sub>/Fc $\epsilon$ RII<sub>b</sub>) were identified (20, 24-26). A comparison of the corresponding cDNA revealed a difference of six amino acids at the cytoplasmic N-terminus of the two Fc $\epsilon$ RII which could be related to their different biologic functions. The Fc $\epsilon$ RII<sub>a</sub> is expressed in normal B cells and certain B cell lines, whereas the Fc $\epsilon$ RII<sub>b</sub> is detectable in several leukocytes, whereas the Fc $\epsilon$ RII<sub>b</sub> is detectable in several leukocytes, whereas the Fc $\epsilon$ RII<sub>b</sub> is not found on normal B lymphocytes and monocytes, it can be induced by IL-4. Interestingly, the Fc $\epsilon$ RII<sub>b</sub> is also expressed on PBL of atopic patients.

We have previously cloned the genomic gene coding for the human Fc $\epsilon$ RII (27). To gain more insight into the regulation of the Fc $\epsilon$ RII-gene expression, we now have functionally analyzed the B cell specific promoter which is regulating the Fc $\epsilon$ RII<sub>a</sub> expression. Regulatory elements were defined in the 5'-flanking region of the Fc $\epsilon$ RII<sub>a</sub> promoter by transiently expressing fusion genes where different portions of the Fc $\epsilon$ RII<sub>a</sub> promoter and a CAT<sup>3</sup> reporter gene were combined. Jijoye cells, a Burkitt lymphoma B cell line, have been chosen as target cells for our studies, because the expression of the Fc $\epsilon$ RII in these cells is known to be induced by IL-4 (28, 29).

### MATERIALS AND METHODS

**Reagents.** DEAE Dextran (m.w. ~ 500,000) was obtained from Pharmacia (Uppsala, Sweden). Chloroquine and acetyl coenzyme A were purchased from Sigma Chemical Co. (St. Louis, MO). <sup>14</sup>C-chloramphenicol in 0.25 M Tris-HCl pH 7.5 was from Amersham (Braunschweig, West Germany). Restriction endonucleases were from Boehringer (Mannheim, West Germany) and used as recommended by the supplier. A Bluescript-Exo/Mung kit was obtained from Stratagene (San Diego, CA). RPMI 1640 was purchased from Wtaker Bioproducts (Walkerville, MD). DMEM was from Flow Laboratories (Herts, England).  $\alpha$ -MEM, FCS, L-glutamine, and gentamicin (10 mg/ml) were received from Gibco (Paisley, Scotland).

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<sup>3</sup> Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; Fc $\epsilon$ RII, lymphocyte receptor for IgE or CD23 Ag; IgE-BF, IgE-binding factor or soluble CD23.

Human rIL-4 was used as a supernatant of stably transformed Chinese hamster ovary cells (H. Hofstetter, manuscript in preparation). The IL-4 titer was 300,000 U/ml, using a commercially available rIL-4 preparation obtained from Genzyme (Boston, MA) as reference.

**Maintenance of cells.** Jijoye cells, obtained from the American Tissue Culture Collection (Rockville, MD), were cultured in complete culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 100 µg/ml gentamicin).

**Plasmids and sequencing.** The cloning of the 5'-region of the Fc.RII<sub>b</sub> gene was described earlier (27). Extended DNA sequencing was performed using the dideoxy chain-termination method (30). Various fragments of the 5'-flanking region of the Fc.RII<sub>b</sub> gene were prepared using suitable restriction endonucleases and then inserted into the expression vector pCMVcat (31) after removal of the CMV-enhancer/promoter cassette by treatment with XbaI and HindIII endonuclease and filling of the 5'-overhanging ends with Klenow polymerase. Plasmid pUSprocat contains the entire 5'-region of the Fc.RII<sub>b</sub> extending from position -1288 of Figure 1 to position 189 of the Fc.RII<sub>b</sub> cDNA (24). The AUG translation start codon of Fc.RII<sub>b</sub> is located at position 189-191 of the cDNA, and it is therefore missing in all CAT-expression plasmids. Plasmid pUSprocat-2 contains the 5'-region between positions -1288 and 80. Serial deletions were based on the plasmid pUSprocat-2. After digestion with XbaI, the plasmid was shortened using exonuclease III and mung-bean nuclease, as recommended by the supplier. The samples were treated with Klenow polymerase, then XbaI linkers were added. Subsequently, the preparations were digested with XbaI/HindIII and the resected fragments subcloned between the XbaI and HindIII restriction sites of pUSprocat-2. The resulting deletion mutants were checked by restriction-endonuclease analysis and DNA sequencing.

**Transfection of cell lines.** Transfection conditions were optimized using the powerful and versatile expression plasmid pCMVcat (31). Plasmid DNA used for transfection was purified by two banding steps in caesium chloride and ethidium bromide. Jijoye cells were freshly split 16 to 24 h before transfection and seeded at a density

of  $5 \times 10^5$  cells/ml. A total of  $4 \times 10^7$  cells were collected and washed twice with Tris-buffered saline. The cells were suspended in a mixture of 4 µg plasmid DNA, 500 µl DEAE Dextran (5 mg/ml in Tris-buffered saline) and 850 µl Tris-buffered saline supplemented with 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH 6.8. After incubation at room temperature for 30 min, the cells were shocked in complete growth medium containing 0.1 mM chloroquine for 1 h. The cells were then washed with complete growth medium, divided into two aliquots, and cultured in complete growth medium. 0.2% of IL-4 containing Chinese hamster ovary cell supernatant or 0.2% of control supernatant was added 16 h after transfection.

**Analysis of transfected cells.** A total of 40 h after transfections, cellular extracts were prepared and 50 µg of protein were assayed for CAT activity for 16 h (32). After TLC, the radioactive spots were cut out and the radioactivity was determined by liquid scintillation counting.

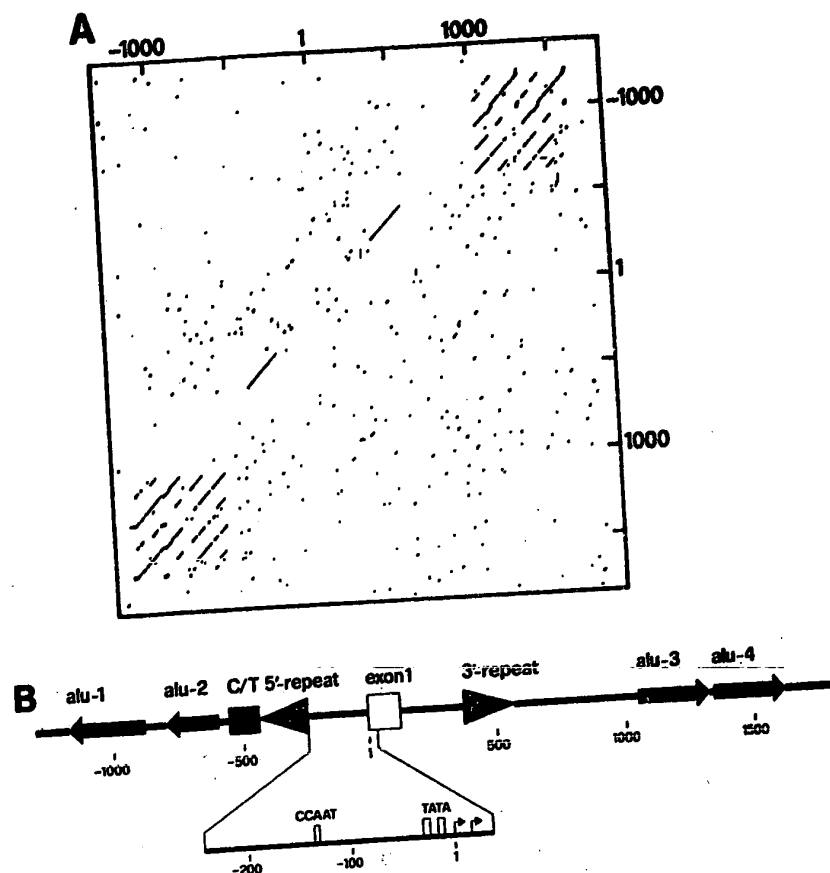
## RESULTS

**Structure of Fc.RII<sub>b</sub> promoter and its flanking region.** The 5'-flanking region of the cloned Fc.RII<sub>b</sub> gene was analyzed by DNA sequencing (Fig. 1) and computer-aided sequence analysis revealed distinctive structural elements within this DNA segment (Fig. 2). The most striking features consist of two pairs of Alu sequences (33) separated by 1600 bp. They are arranged as an inverted repeat surrounding the Fc.RII<sub>b</sub> core promoter. In addition, two 188-bp long, almost perfectly identical sequence elements forming another inverted repeat were found adjacent to the Alu repeats. One of the 188-bp repeat elements is located about 300 bp 5' of the TATA boxes, whereas its inverted partner is found in the first

-1289 AACCTGGGA ATAGTTCAG CTGGCCATG TTGTAGGAC ATGGGAGGA AGCTGAGT CAGGATGGG GCTGGATAG AGTTGATGT TCTTTCTTC  
-1189 TTTTCTTTC TTTTCTTTC TCTTTTTC TTATAGAGA CAGAGTTAG CTTTCTTTC CAGGCTGGA ATGCAATGC GATCTGTC TACTGTCAC  
-1089 CATGCTTC GAGTTCAG CAGCTTCT GCTCTAGCT CCGAGTAC TAGATGAGA GTGCTGACC AGGCTGCCA GCTATTTTC ATATTTTTC  
-989 TTTTCTTTC GTATTTTC TAGATGAGA GTTCTACAT GTTGTCTTG AACTCTGAC CTCAGATAT CCACTGCTT GAGCTGCCA AGTCTGTCG  
-889 ATGACAGGC CTGAGATGT GCGGCTTC TCTCTCTC TTCTCTCT TCTTCTTC ATCTCACTT GTTGTGCGG GCTATCTTG AACTCTGTCG  
-789 CTGAGTAT CCACTGCTT CTGCTGCTT AGTACTGAG ATCAGAGCA TCACACCA TCCCTGCTA GTTTTATAG TTTTGTAGA GACAGCTCT  
-689 TCTATGTTG TCGAGCTTG TCTGAGTC CAGGCTTAA GTATCTCT CACTGAGC TCCCAATGT CTGAGTAT AGGCTGAC GTTCTGCTA  
-589 GCAATCTGT CCAATTTTA CCAATATAT ATCTCACTA TCTTCTTC CCGGCTTC CCTCTGCT TCCATCTCT CTTCTGCTT CTTCTGCTT  
-489 CTGCTTTC TTGCTCTC CTGCTGCTT CTTCTCTT CTTCTTTC TTCTGCTC TCAAGCTTG GATCTGAC CTGAGCTCT ATTTCTCAA  
-389 TCTAGAGG AATATCTC ATGCTGCT GTTCTGCTT ATGCTGCTT TCTGCTCT CCAATCTCT GCTCTCTC TATGCTGCA GCTCTGCTT  
-289 CTGCTGCTT CTTCTGCTT CTTCTGCTT CTTCTGCTT TCTGCTCT TCTGCTCT TCTGCTCT TCTGCTCT TCTGCTCT TCTGCTCT  
-189 TCACTCTT CAGCTGCTT GAACTGCTT TCTATCTC TCTATCTC AATATCTC TCTATCTG ATATGATAT ATATATCA CAGCTCTAC  
-89 CCGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT TCTATCTC TCTATCTC TCTATCTC TCTATCTC TCTATCTC TCTATCTC  
12 TTTGAGAGA AGTCTCTT CTTCTGCTT AATCTCTT CTTGAGCTT CTTGAGCTT CTTGAGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
112 CTTCTGCTT CCAATCTG ATATCTAT TCTGCTT GGGTGTCT CAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
212 CTTCTGCTT ATATCTAT GAGCTGCTT CAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
312 CAGCTGCTT CCAATCTG GAGCTGCTT CAGCTGCTT TCTGCTCT TCTGCTCT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
412 GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
512 TCTGCTCT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
612 TCTGCTCT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
712 TCTGCTCT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
812 ATGCTGCTT TCTGCTCT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
912 CTTCTGCTT GAGCTGCTT CCAATCTG ATATCTAT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
1012 GTAGCTCT TATGCTCT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
1112 TCTGCTCT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
1212 GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
1312 CCAATCTG GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
1412 TCTGCTCT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
1512 GAGCTGCTT TCTGCTCT CAGCTGCTT GAGCTGCTT TCTGCTCT TCTGCTCT TCTGCTCT TCTGCTCT TCTGCTCT TCTGCTCT  
1612 ATATCTAT GAGCTGCTT TCTGCTCT TCTGCTCT CAGCTGCTT TCTGCTCT TCTGCTCT TCTGCTCT TCTGCTCT TCTGCTCT  
1712 ATGCTGCTT GAGCTGCTT ATATCTAT TATGCTCT GAGCTGCTT CAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT GAGCTGCTT  
1812 ATGCTGCTT

Figure 1. Nucleotide sequence of the 5'-region of the Fc.RII<sub>b</sub> gene. Solid lines, TATA boxes and CCAAT sequence; thin lines, Alu sequences; dotted line, pyrimidine stretch; boxed sequences, inverted repeat; dots, putative cap site (27); star, 3'-end of first exon.

**Figure 2.** Extended repeat structure surrounding the Fc $\gamma$ RII<sub>a</sub> promoter. **a.** Dot matrix plot comparing the nucleotide sequence of the 5'-region of the Fc $\gamma$ RII<sub>a</sub> gene to itself. Dots indicate positions at which more than 14 nucleotides in a stretch of 21 are identical. Numbering of nucleotides is from Figure 1. **b.** Schematic diagram of the Fc $\gamma$ RII<sub>a</sub> 5'-region summarizing the arrangement of the repeated sequence. C/T, pyrimidine stretch.



intron about 400 bp downstream of the transcription-initiation sites. The upstream 188-bp repeat element and the corresponding Alu sequences are separated by a stretch of 109 consecutive pyrimidines, interrupted by only five purines. Together, the four Alu sequences and the two 188 bp-repeat elements form an extended, nearly symmetrical structure which frames the 5'-regulatory region of the Fc $\gamma$ RII<sub>a</sub> gene.

**Identification of Fc $\gamma$ RII<sub>a</sub> promoter and functional analysis of its flanking regions.** The entire 5'-region of the Fc $\gamma$ RII<sub>a</sub> gene was fused to a bacterial CAT gene and cloned into a mammalian expression vector to yield the plasmid pUSprocat. Several mutants with partially or completely deleted repeats were constructed based on pUSprocat to assess the function of the unusual structural elements in the vicinity of the Fc $\gamma$ RII<sub>a</sub> promoter (Fig. 3). Subsequently, the plasmids were transfected in JIjoye cells for transient expression. All plasmids that contain parts of the repetitive promoter structures show a low level of CAT expression. Only pUSprocat-5 which lacks all these elements is well expressed.

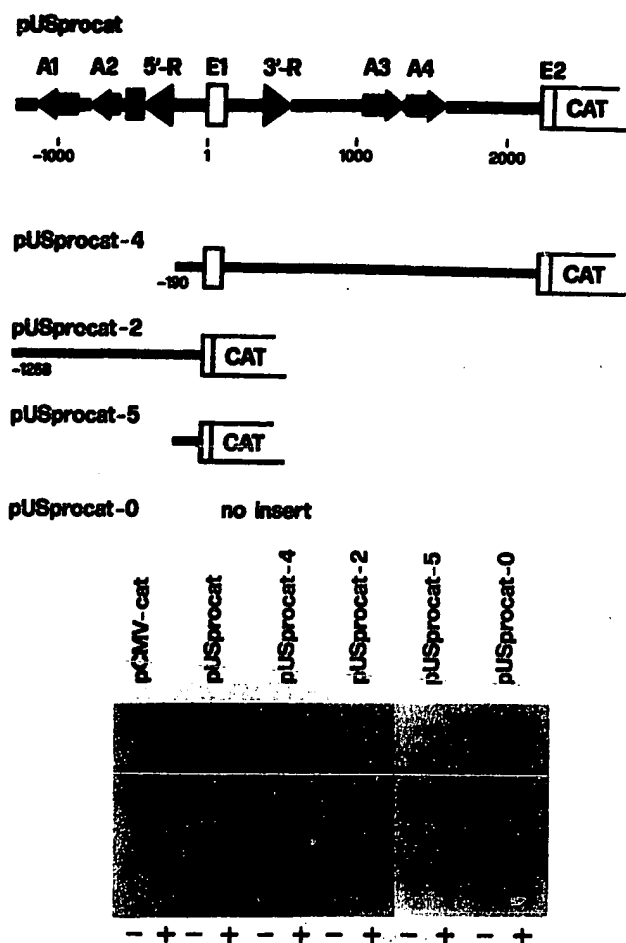
**Fine mapping of regulatory sequences.** Serial deletions in the 5'-flanking region of the Fc $\gamma$ RII<sub>a</sub> gene were assembled based on pUSprocat-2 as the starting plasmid (Fig. 4). Transient expression in JIjoye cells identified two putative regulatory elements. The CAT activity increases about five-fold upon removal of the sequences between -586 (pGTpro-5) and -484 (pGTpro-6), suggesting the presence of a negative regulatory element within this region that consists mainly of pyrimidines. Further dele-

tions up to position -190 (pUSprocat-5) did not change the CAT activity. However, if the segment between -190 and -130 (pGTpro-9) was removed, the promoter activity decreased sharply which indicates the presence of a positive regulatory element. Interestingly, a CCAAT-box consensus sequence (-136 to -132), a motif often involved in the regulation of RNA polymerase II promoters (34), is present in the deleted DNA element.

Addition of IL-4 enhanced CAT expression about two- to four-fold suggesting that an IL-4 responsive sequence element which is present within the Fc $\gamma$ RII<sub>a</sub> promoter region is mediating the IL-4 effect. To locate this regulatory element, the DNA segment from position -253 to -43 of the Fc $\gamma$ RII<sub>a</sub> gene was introduced in front of a thymidine kinase core promoter (35). This sequence element could indeed confer induction by IL-4 onto the thymidine kinase core promoter, which by itself was unaffected by this lymphokine (Fig. 5).

#### DISCUSSION

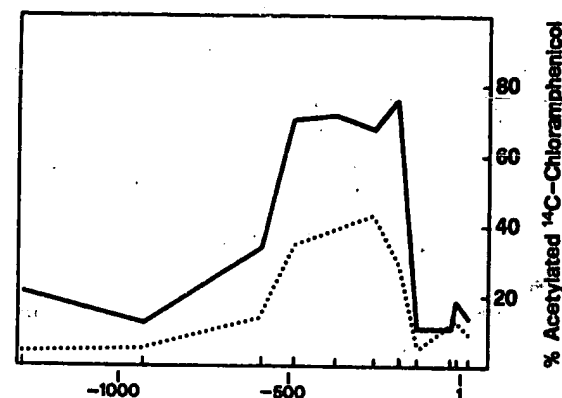
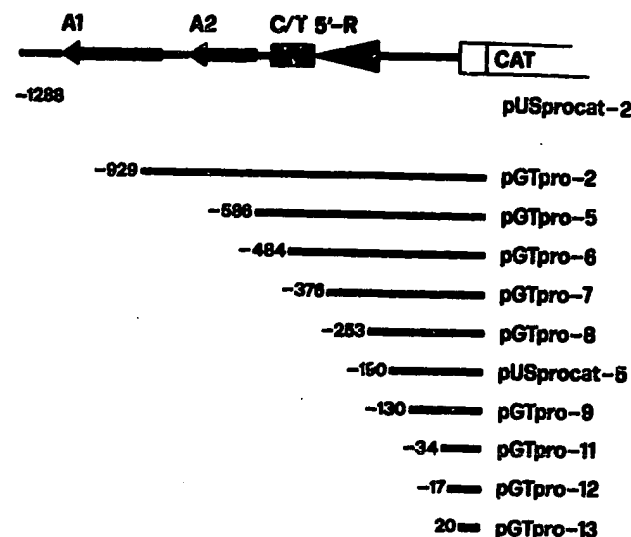
Our analysis of the 5'-region of the Fc $\gamma$ RII<sub>a</sub> gene revealed a striking cluster of four Alu sequences and two 188-bp repeat elements that frame the Fc $\gamma$ RII<sub>a</sub> core promoter. Alu sequences are highly redundant and scattered throughout the human genome (33). They are found mostly between genes, sometimes in introns and very rarely in coding sequences (36). In contrast, the 188-bp repeat element seems to be restricted to the Fc $\gamma$ RII<sub>a</sub> promoter. We could not find any cross-hybridization to human genomic DNA using conditions sufficient to detect



**Figure 3.** Functional analysis of the 5'-region of the Fc $\gamma$ RII<sub>b</sub> gene. *a*, Diagram of wt and deletion mutants of the Fc $\gamma$ RII<sub>b</sub> 5'-region fused to the CAT structural gene. Characteristic sequence elements are shown for the wt (pUSprocat), corresponding sequences present in the deletion mutants are indicated with thick lines. *b*, Effect of deletions on expression of CAT activity in Jijoye cells transiently transfected with the plasmids shown in *a*. + and - indicate addition and omission of IL-4, respectively. Five independent transfection experiments have been performed and the result of a representative one is shown.

DNA-sequence similarities higher than 90% and the 188-bp repeat element as a probe (data not shown). The four Alu sequences and the two 188-bp repeat elements constitute an extended inverted repeat surrounding the Fc $\gamma$ RII<sub>b</sub> promoter. However, their functional role remains unknown as the completely intact promoter region had a strongly inhibitory effect on transcription in our assay system. Bearing in mind that the Fc $\gamma$ RII is only expressed in mature  $\mu^+$  $\delta^+$  B lymphocytes, but absent on B cell precursors as well as on B cells after isotype switching (16), the repetitive structure could possibly form a large stem-loop in vivo which might be involved in controlling the restricted expression of the Fc $\gamma$ RII<sub>b</sub> during B cell development (16). Interestingly, in the HLA-DR $\alpha$  gene whose expression is regulated coordinately with the Fc $\gamma$ RII (28), the promoter is also flanked by two Alu sequences at about the same distance from the transcriptional initiation site as found in the Fc $\gamma$ RII<sub>b</sub> gene (37).

The core promoter of the Fc $\gamma$ RII<sub>b</sub> gene was identified from position -190 to +80 within the 5'-flanking region. If the sequence between -190 and -130 is removed, the promoter activity is strongly decreased. The deleted DNA segment includes a CCAAT-box, a sequence motif that



**Figure 4.** Fine mapping of regulatory sequences in the 5'-flanking region of the Fc $\gamma$ RII<sub>b</sub> gene. *a*, Schematic representation of wt and deletion mutants. *b*, CAT activity measured in Jijoye cells transiently transfected with wt and mutant plasmids. Continuous line, addition of IL-4; dotted line, without IL-4.

binds several known transcription factors (38) and that was recently shown to be involved in B cell specific gene regulation (39). In addition, a negative regulatory element consisting of >95% pyrimidines was mapped further upstream in the promoter region. Such segments are often regulating tightly controlled genes such as c-myc (40).

The expression of the Fc $\gamma$ RII<sub>b</sub> on B cells is inducible by IL-4 (16). Inasmuch as freshly isolated B lymphocytes are not easily amenable to DNA transfection, we used Jijoye cells as a model system. Jijoye cells have been shown to bear IL-4R on their surface (41) and their Fc $\gamma$ RII expression is augmented after the addition of IL-4 (28). We have located the DNA element responding to IL-4 within 250-bp upstream of the TATA box. Although the amplitude of the IL-4-dependent transcriptional activation of the transfected genes was small, it corresponds to the moderate changes seen at the steady-state mRNA level of the endogenous Fc $\gamma$ RII gene. Yokota et al. (28) have shown that the Fc $\gamma$ RII, as well as the Fc $\gamma$ RII<sub>b</sub> mRNA are induced by IL-4. This finding, strongly supported by the results of our studies, suggests common DNA elements involved in the IL-4-mediated up-regulation of the two Fc $\gamma$ RII promoters. Such a DNA segment that binds an IL-4 regu-

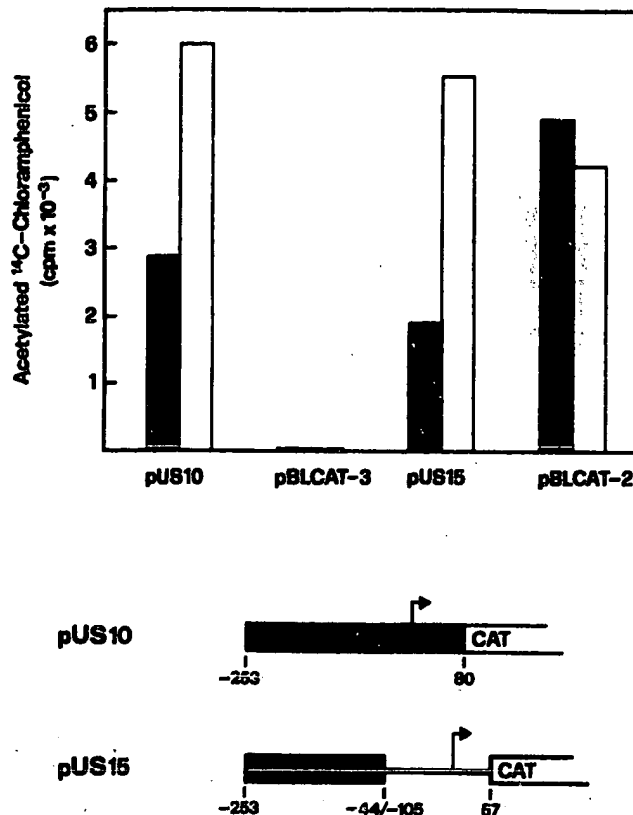


Figure 5. Identification of an IL-4 responsive element. Plasmid pUS10 was derived from plasmid pBLCAT3 (35) by insertion of the Fc $\gamma$ RII core promoter (positions -253 to +80 of Fig. 1) into the XhoI restriction site upstream of the CAT gene. Plasmid pUS15 was constructed by insertion of Fc $\gamma$ RII sequences (positions -253 to -43) between the XbaI and BamHI restriction sites of plasmid pBLCAT2 (35). The diagram shows the CAT activity measured after transient transfection of Jijoye cells.

lated, putative transcription factor has recently been found in the regulatory region of a MHC-II gene (42).

IL-4 and the Fc $\gamma$ RII are thought to play key roles in the development of allergies. This view is supported by the parallel enhancement of IgE and Fc $\gamma$ RII expression by IL-4 (23, 43). Furthermore, Fc $\gamma$ RII-specific mAb block the in vitro IgE synthesis in an isotype-specific manner. The study of Fc $\gamma$ RII-gene regulation provides a valuable model to determine IL-4-mediated molecular events with the final goal of gaining a better understanding of IgE regulation.

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## DIFFERENTIAL EXPRESSION OF Fc $\gamma$ RIIA, Fc $\gamma$ RIIB AND Fc $\gamma$ RIIC IN HEMATOPOIETIC CELLS: ANALYSIS OF TRANSCRIPTS

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**Abstract**—Fc $\gamma$  receptors (Fc $\gamma$ R) are glycoproteins that function in the immune response through their ability to bind the Fc portion of immunoglobulin G. Of the three human Fc $\gamma$ R classes, Fc $\gamma$ RII is most widely distributed among hematopoietic cells and is the only Fc $\gamma$ R class present on platelets and megakaryocytes. There are three different genes coding for Fc $\gamma$ RII: Fc $\gamma$ RIIA, Fc $\gamma$ RIIB and Fc $\gamma$ RIIC. Alternative splicing of at least two of these genes results in the production of multiple transcripts. Combining Northern blot analysis with reverse transcription-PCR, we analyzed steady state levels of Fc $\gamma$ RII mRNA in the megakaryocytic, myeloid and lymphoid lineages. We determined that megakaryocytic cells predominantly contain Fc $\gamma$ RIIA mRNA; Fc $\gamma$ RIIA transcripts with and without the transmembrane exon (Fc $\gamma$ RIIA1 and Fc $\gamma$ RIIA2, respectively) are present in comparable amounts. In contrast, B lymphocytes do not express Fc $\gamma$ RIIA mRNAs, but do contain both Fc $\gamma$ RIIB transcripts, Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2, as well as the Fc $\gamma$ RIIC transcript, Fc $\gamma$ RIIC. Myelomonocytic cells contain mRNAs from all three Fc $\gamma$ RII genes, predominantly the Fc $\gamma$ RIIA1 transcript, both Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2 transcripts and Fc $\gamma$ RIIC. Lineage-specific expression of the Fc $\gamma$ RII genes implies both differential regulation of expression and differential function in diverse cells.

### INTRODUCTION

The Fc $\gamma$  receptors (Fc $\gamma$ R) are glycoproteins which bind the Fc structure of immunoglobulin G and, thus, link the humoral and cellular components of the immune system. Three biochemically distinct classes of Fc $\gamma$ R have been described: Fc $\gamma$ RI (CD64), Fc $\gamma$ RII (CD32) and Fc $\gamma$ RIII (CD16). Fc $\gamma$ RII is present with other Fc $\gamma$ R classes on the surface of monocytes, macrophages, and granulocytes, while it is the only Fc $\gamma$  receptor class present on platelets, megakaryocytes and B lymphocytes (Anderson and Looney, 1986; Unkeless, 1989; Schreiber *et al.*, 1989; King *et al.*, 1990; Sarmay *et al.*, 1990; Gewirtz *et al.*, 1992), the megakaryocytic cell line, HEL (King *et al.*, 1992) and the multipotential hematopoietic cell line K562 (Tax and van de Winkel, 1990). Human Fc $\gamma$ RII cDNA clones isolated from myeloid, lymphoid and megakaryocytic cell lines show structural heterogeneity, and three different genes coding for Fc $\gamma$ RII have been identified and isolated: Fc $\gamma$ RIIA, Fc $\gamma$ RIIB and Fc $\gamma$ RIIC (Brooks *et al.*, 1989; Qiu *et al.*, 1990). The cDNA structure for the Fc $\gamma$ RII class consists of a 5'-untranslated region, sequences coding for a signal peptide region (S), an extracellular domain (EC) con-

taining two loops with homology to members of the immunoglobulin gene superfamily, a single transmembrane region (TM), an intracytoplasmic domain (C) and a 3'-untranslated region. Fc $\gamma$ RIIA, Fc $\gamma$ RIIB and Fc $\gamma$ RIIC are highly homologous in their extracellular domains, although Fc $\gamma$ RIIA has two sites of N-linked glycosylation while Fc $\gamma$ RIIB and Fc $\gamma$ RIIC have three. Fc $\gamma$ RIIB differs considerably from Fc $\gamma$ RIIA and Fc $\gamma$ RIIC in the intracytoplasmic domain, sharing only the first eight amino acids.

Not only are there three Fc $\gamma$ RII genes, but there are also multiple Fc $\gamma$ RII transcripts in hematopoietic cells, as demonstrated by cDNA cloning (Walterhouse *et al.*, 1988; Brooks *et al.*, 1989; Seki, 1989; Stuart *et al.*, 1989; Warmerdam *et al.*, 1990). These transcripts include Fc $\gamma$ RIIA1, Fc $\gamma$ RIIA2, Fc $\gamma$ RIIB1, Fc $\gamma$ RIIB2, Fc $\gamma$ RIIB3 and Fc $\gamma$ RIIC. The 3'-untranslated region of Fc $\gamma$ RIIA has two alternative polyadenylation sites 1 kb apart which result in 1.6 and 2.6 kb transcripts, while Fc $\gamma$ RIIB and Fc $\gamma$ RIIC have single polyadenylation sites encoding only 1.6 kb transcripts. We previously reported the cloning of an Fc $\gamma$ RIIA cDNA which lacked the transmembrane region from HEL cells (Walterhouse *et al.*, 1988), and demonstrated the existence of the corresponding mRNA, Fc $\gamma$ RIIA2, in HEL, CHRF-288-11 and U937 cells, and platelets (Rappaport *et al.*, submitted). This mRNA suggests the existence of a soluble form of the Fc $\gamma$ RIIA protein. A similar transcript was found in K562 cells (Warmerdam *et al.*, 1990).

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We have also demonstrated that the Fc $\gamma$ RIIA primary transcript can undergo alternative splicing in the 5'-untranslated region (McKenzie *et al.*, 1992). Three transcripts of Fc $\gamma$ RIIB (Fc $\gamma$ RIIB1, Fc $\gamma$ RIIB2, Fc $\gamma$ RIIB3) have been characterized, which differ due to alternative splicing of the second signal peptide exon (S2) and the first cytoplasmic exon (C1) (Brooks *et al.*, 1989).

It is not known which Fc $\gamma$ RII genes are transcribed in each blood cell lineage, nor is it known which transcripts are present. Antibodies which distinguish the different Fc $\gamma$ RII proteins have not been developed. Definition of the distribution and gene origin of the Fc $\gamma$ RII transcripts is crucial for studies of both transcriptional regulation and post-transcriptional processing. Knowledge of transcript distribution is also important to analyze the function of the individual transcripts. In this study we analyzed RNA from both primary cells and cell lines of various hematopoietic lineages for the presence of Fc $\gamma$ RII transcripts. We used both Northern blot analysis with Fc $\gamma$ RII gene-specific probes and reverse transcription-PCR (RT-PCR) using Fc $\gamma$ RII gene-specific primer pairs to determine steady state Fc $\gamma$ RII mRNA levels in different hematopoietic cell lineages.

## MATERIALS AND METHODS

### Cells

The human megakaryocytic cell line, HEL (ATCC TIB 180); the human monocytic cell line, U937 (ATCC CRL 1593); the human multipotential hematopoietic cell line, K562 (ATCC CCL 243); and the human B lymphocyte cell lines, Raji (ATCC CCL 86) and RPMI 1788 (ATCC CCL 156) were obtained from the American Type Culture Collection (Rockville, MD). The human megakaryocytic cell line, CHRF-288-11, was kindly provided by Dr David P. Witte (Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH) (Fugman *et al.*, 1990). All of these cell lines were maintained in RPMI 1640 (Sigma Chemical Company, St. Louis, MO) supplemented with 10% (v/v) Serum Plus (Hazelton Biologics, Inc., Lenexa, KS) and with 2 mM L-glutamine, 1  $\times$  BME Vitamin Solution and 100 units penicillin/100 mg streptomycin per ml (Gibco BRL, Gaithersburg, MD). The human megakaryocytic cell line Dami was obtained from Dr Sheryl Greenberg (Department of Medicine, Harvard Medical School, Boston, MA) (Greenberg *et al.*, 1988). Dami cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 10% (v/v) horse serum (Sigma Chemical Company) and with 2 mM L-glutamine and 100 units penicillin/100 mg streptomycin (Gibco BRL) per ml.

Human platelets were isolated from platelet-rich plasma (PRP) obtained by differential centrifugation of whole blood from normal donors. The PRP had a white cell to platelet ratio of approximately 1:1000. Human granulocytes were isolated by centrifugation through LSM (Organon-Teknika, West Chester, PA) and human monocytes were isolated by adherence to serum-treated

flasks as described previously (Darby *et al.*, 1990; Chien *et al.*, 1988).

### cDNA clones

A full length human Fc $\gamma$ RIIA cDNA (HFc3.0), corresponding to the Fc $\gamma$ RIIA transcript, was kindly supplied by P. Mark Hogarth (University of Melbourne, Victoria, Australia) (Hibbs *et al.*, 1988). Human Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2 cDNAs were kindly provided by Jeffrey V. Ravetch (Memorial Sloan-Kettering Cancer Research Institute, New York, NY) (Brooks *et al.*, 1989).

### RNA isolation

RNA was prepared by dispersal in guanidinium thiocyanate and extraction with cold acid phenol (Chomczynski and Sacchi, 1987). The final pellet of RNA was resuspended in diethylpyrocarbonate-treated water and stored at  $-70^{\circ}\text{C}$ . RNA concns were determined spectrophotometrically, except for platelet preparations, for which amounts to be used were estimated from electrophoresis on non-denaturing agarose gels. RNA quality was assessed by electrophoresis on agarose gels.

### Northern blot analysis

Thirty micrograms of total RNA was separated on denaturing 1% (w/v) agarose/formaldehyde gels following standard procedures (Sambrook *et al.*, 1989). Capillary transfer of RNA to Zetabind membranes (Cuno, Inc., Meriden, CN) was carried out in 1  $\times$  HETS buffer (TM Cinna Scientific, Inc., Friendswood, TX) overnight. Blots were dried at  $55^{\circ}\text{C}$  for 30 min and the transferred RNA was UV-crosslinked to the membrane in a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The blots were washed in  $0.2 \times \text{SSC}$ , 0.5% (w/v) SDS for 30 min at  $68^{\circ}\text{C}$  to prevent non-specific binding of radioactive probe. The blots were prehybridized at  $68^{\circ}\text{C}$  for at least 1 hr in hybridization buffer containing: 0.5 M sodium phosphate, pH 7.5; 7% (w/v) SDS; 1% (w/v) BSA; 1 mM EDTA; heat denatured salmon sperm DNA (50  $\mu\text{g}/\text{ml}$ ); and polyadenylic acid (10  $\mu\text{g}/\text{ml}$ ) (Sigma).  $^{32}\text{P}$ -labeled probes (see below) ( $5\text{--}10 \times 10^6$  dpm/ml, 0.1–0.6 pmol/ml) were added and the blots hybridized at  $68^{\circ}\text{C}$  for 16 hr. Blots were washed twice at room temp with  $0.5 \times \text{SSC}$ , 0.1% (w/v) SDS for 1 min and once or twice at  $63^{\circ}\text{C}$  with the same solution for 15 min. Washed blots were exposed to Kodak X-OMAT-AR film at  $-70^{\circ}\text{C}$  for 1–4 days. Blots were stripped for reprobing by washing in boiling water.

### Reverse transcription-polymerase chain reaction (RT-PCR) amplification

(a) *cDNA synthesis.* First-strand cDNA was synthesized from 10  $\mu\text{g}$  total cell RNA (or 2  $\mu\text{g}$  platelet RNA) using Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Gibco BRL, Grand Island, NY) in a total volume of 20  $\mu\text{l}$  by a modification of a procedure previously described (Witsel and Schok, 1990). The first-strand cDNA reaction mix contained 1.3  $\mu\text{M}$  Random Primer<sup>2</sup> (Promega, Madison, WI);

500 μM each dATP, dCTP, dGTP, TTP (Ultrapure dNTP Set, Pharmacia, Piscataway, NJ); 1 × RT Buffer (BRL/Life Technologies), 10 mM dithiothreitol and 500 units M-MLV RT. 1 × RT Buffer is 50 mM Tris-HCl, pH 8.3; 75 mM KCl and 3 mM MgCl<sub>2</sub>. The reaction mixture was held at room temp for 10 min, incubated at 42°C for 1 hr and then heated at 90°C for 10 min to terminate. The reaction mixture was diluted 1:2 by the addition of 20 μl of 1 × RT Buffer for a final volume of 40 μl.

(b) *Amplification of cDNA by PCR.* Twelve microliters of the diluted cDNA reaction mix were amplified in a total volume of 100 μl containing 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM MgCl<sub>2</sub>; 0.001% (w/v) gelatin; 200 μM each dATP, dCTP, dGTP, TTP; 1 μM 5'-primer (sense); 1 μM 3'-primer (antisense) and 1.5 units Ampli-Taq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT). Fifty microliters of paraffin oil were placed on top of the reaction to prevent evaporation. The mixture was amplified for 40 cycles using a Perkin-Elmer Cetus Thermal Cycler. Each cycle consisted of a denaturation step (94°C for 1.0 min), an annealing step (60°C for 1.5 min) and an elongation step (72°C for 1.0 min) in that order. Alternatively, the mixture was amplified for 25 cycles using a Perkin-Elmer Cetus GeneAmp PCR System 9600. Each cycle consisted of a denaturation step (94°C for 30 sec), an annealing step (64°C for 30 sec) and an elongation step (72°C for 1.0 min) in that order.

#### Analysis of PCR Products

Ten microliters aliquots of the PCR reaction mixtures were analyzed by gel electrophoresis on 1.5% (w/v) agarose gels for size determination. Southern blotting to Zetabind membranes (Cuno, Inc., Meriden, CN) was performed according to the manufacturer's specifications. The membranes were pre-hybridized in 6 × SSC; 50 mM sodium phosphate, pH 6.8; 5 × Denhardt's reagent; 100 μg/ml heat-denatured salmon sperm DNA for at least 1 hr, and then hybridized for 16 hr at 37°C in the same solution containing 1 × 10<sup>5</sup> cpm/ml of labeled oligonucleotide probe. Blots were washed once at room temp for 15 min and once at 50°C for 15 min with 6 ×

SSC, 0.05% (w/v) sodium pyrophosphate and exposed to Cronex film (Dupont, Wilmington, DE) at room temp for 1–5 hr. Blots were stripped for reprobing by washing once with 200 ml of 0.4 M sodium hydroxide for 15 min at 42°C followed by once with 200 ml of 0.1 × SSC, 0.5% (w/v) SDS, 0.2 M Tris-HCl (pH 7.5) for 15 min at 42°C.

#### Preparation of oligonucleotides primers and hybridization probes

Oligonucleotides were synthesized on a DNA Synthesizer 380B (Applied Biosystems, Inc., Foster City, CA). The sequence, gene origin and gene location of these oligonucleotides are shown in Table 1 and Fig. 2. The gene-specific primer pairs used for PCR were: for the FcγRIIA gene, 1S and 224M; for the FcγRIIB gene, 2S and 241M; and for the FcγRIIC gene, 2S and 224M. Oligonucleotides used as hybridization probes were kinase-labeled according to standard procedures (Sambrook *et al.*, 1989). Hybridization probes used for the analysis of RT-PCR products included 16M, 17M, TM, DL and CI. Hybridization probes for Northern blot analysis were obtained using PCR with primers specific for either the FcγRIIA or the FcγRIIB/IIIC first signal exon. The primer pair used to synthesize the 87 bp FcγRIIA-specific probe from the FcγRIIA cDNA (Fc3.0) was 1SS and 28M. The primer pair used to prepare the 109 bp FcγRIIB/IIIC-specific probe from the FcγRIIB cDNA was 2S and 36M. One nanogram of FcγRII cDNA was amplified by PCR in a total volume of 100 μl as described above. The PCR products were purified from agarose gels using a GeneClean II Kit (Bio 101, La Jolla, CA). One nanogram of the cold PCR product was radioactively labeled by PCR in a total volume of 25 μl as described above except <sup>32</sup>P-α-dCTP and <sup>32</sup>P-α-dATP (100 μCi, 1.3 μM final concn each) were substituted for unlabeled dCTP and dATP. The labeled oligonucleotide probes were purified by passage over a Sephadex G-50 column (Sigma Chemical Company), and their quality and quantity determined by agarose gel chromatography followed by autoradiography and by scintillation counting.

Table 1. Sequences of oligonucleotides

Name	Sequence		
1S <sup>a</sup>	5'-ATG	TCT CAG AAT GTA TGT CCC	AGA-3'
224M <sup>b</sup>	5'-CTC	AAA TTG GGC AGC CTT CAC-3'	
2S	5'-GGA	ATC CTG TCA TTC TTA CCT	GTC-3'
241M	5'-CCC	AAC TTT GTC AGC CTC ATC-3'	
16M	5'-CAA	TGG TTG AAG CCA CAG GTT-3'	
17M	5'-AGC	CCA GTC ACT CTC AGT GGC	AAG-3'
TM	5'-CCA	ATG GGG ATC ATT GTG GC-3'	
DL	5'-TGG	AAT TGG CTT GGA CAG TG-3'	
CI	5'-GTT	TCT CAG GGA GGG TCT CT-3'	
1SS	5'-GGA	TGA CTA TGG AGA CCC AAA	TG-3'
28M	5'-AGC	AGC AGC AAA ACT GTC AAT	GG-3'
36M	5'-CCA	GGA ATA GCA CAG CTG TCC	ACA-3'

<sup>a</sup>S indicates a sense primer.

<sup>b</sup>M indicates an antisense primer.

## RESULTS

(1) *FcγRIIA* transcript distribution

(a) *FcγRIIA* mRNAs are the predominant *FcγRII* transcripts in megakaryocytic cells. We used a probe specific for *FcγRIIA* and a probe recognizing both *FcγRIIB* and *FcγRIIC* (*FcγRIIB/IIIC*) but not *FcγRIIA* for Northern blot analysis to determine steady state *FcγRII* mRNA levels in different megakaryocytic cell lines. *FcγRIIB* and *FcγRIIC* transcripts are not distinguished using this approach. *FcγRIIA* transcripts (1.6 and 2.6 kb) were found in all three megakaryocytic cell lines tested (Dami, CHRF-288-11, HEL; Fig. 1A, lanes 2-4). To determine the transcripts present in primary cells, we analyzed platelet RNA by RT-PCR with *FcγRIIA*-*FcγRIIB*- and *FcγRIIC*-specific primer pairs (Fig. 2). Although the three *FcγRII* genes are highly homologous, *FcγRIIA*

has unique signal exons and *FcγRIIB* has unique cytoplasmic exons. *FcγRIIC* shares sequence homology with *FcγRIIB* signal exons and *FcγRIIA* cytoplasmic exons. This allowed us to use pairs of primers specific for each of the three *FcγRII* genes. Each pair consisted of a primer complementary to a region in the first signal exon and a second primer complementary to a region in the first or second cytoplasmic exon. PCR amplification of platelet RNA with *FcγRIIA*-specific primers yields 762 bp and 639 bp bands (Fig. 3, lanes 3 and 4). Likewise, PCR amplification of HEL cell RNA with *FcγRIIA*-specific primers yields 762 bp and 639 bp bands (Fig. 5A, lane 2). Southern blot analysis of the platelet PCR products demonstrates that the 762 bp band arises from the *FcγRIIA1* transcript containing the TM exon and the 639 bp band arises from the *FcγRIIA2* transcript lacking this exon (Fig. 4); HEL cell PCR

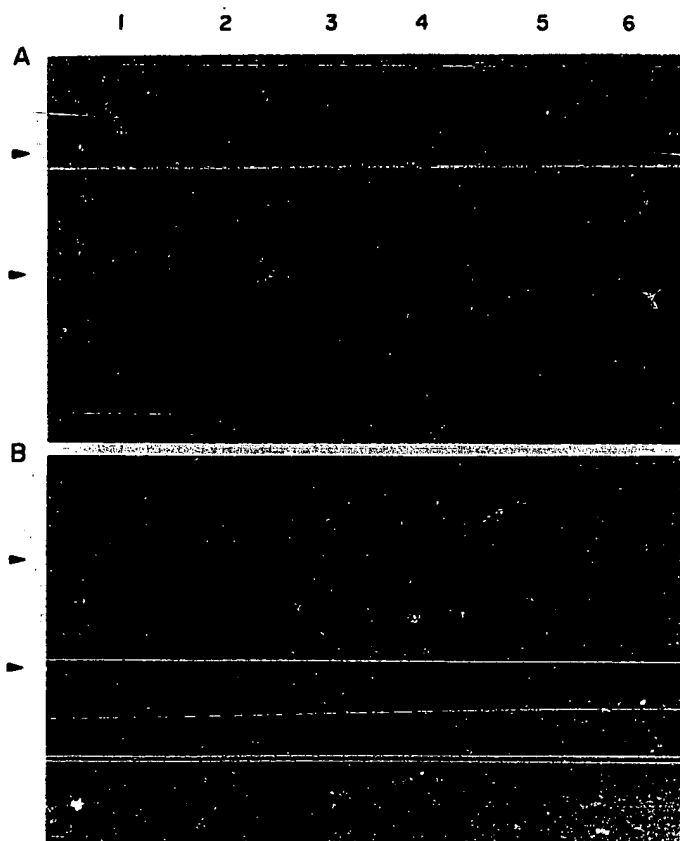


Fig. 1. Northern blot analysis of hematopoietic cell lines. (A) A Northern blot containing RNA from several different hematopoietic cell lines was hybridized with the 87 bp PCR-generated probe specific for *FcγRIIA*. Myeloid (lanes 1 and 5), megakaryocytic (lanes 2-4) and B lymphocytic (lane 6) cell line RNAs were analyzed. Each lane contains 30  $\mu$ g of total RNA. Lane 1, K562; lane 2, Dami; lane 3, CHRF-288-11; lane 4, HEL; lane 5, U937; lane 6, RPMI 1788. The upper and lower arrowheads indicate the 2.6 and 1.6 kb transcript sizes, respectively. Lanes 1-5, containing myeloid or megakaryocytic cell line RNAs, have 1.6 and 2.6 kb bands. The B lymphocytic cell line RNA, in lane 6, does not have the 2.6 or 1.6 kb bands, but is positive with an oligonucleotide probe to 18S rRNA (data not shown). (B) A Northern blot containing RNA from several different hematopoietic cell lines was hybridized with the 109 bp PCR-generated probe specific for *FcγRIIB/IIIC*. Myeloid (lanes 4 and 5), megakaryocytic (lanes 1, 2 and 3) and B lymphocytic (lane 6) cell line RNAs were analyzed. Each lane contains 30  $\mu$ g of total RNA. Lane 1, HEL; lane 2, CHRF-288-11; lane 3, Dami; lane 4, K562; lane 5, U937; lane 6, RPMI 1788. Myeloid and B lymphocytic cell line RNAs, in lanes 4, 5 and 6, have 1.6 kb bands. Megakaryocytic cell line RNAs, in lanes 1, 2 and 3, do not have 1.6 kb bands, but are positive with an oligonucleotide probe to 18S rRNA (data not shown).

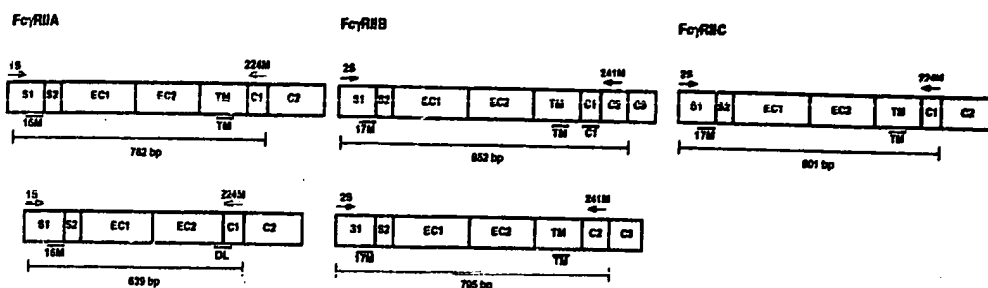


Fig. 2. Strategy for Fc $\gamma$ RII RT-PCR and Southern blot analysis. The structure of the Fc $\gamma$ RIIA, Fc $\gamma$ RIIB and Fc $\gamma$ RIIC cDNAs is shown. The exons are depicted as boxes with names contained inside: S1, first signal exon; S2, second signal exon; EC1, first extracellular exon; EC2, second extracellular exon; TM, transmembrane exon; C1, first intracytoplasmic exon; C2, second intracytoplasmic exon; C3, third intracytoplasmic exon. The upper Fc $\gamma$ RIIA cDNA is Fc $\gamma$ RIIA1; the lower is Fc $\gamma$ RIIA2. The upper Fc $\gamma$ RIIB cDNA is the Fc $\gamma$ RIIB1 transcript; the lower is the Fc $\gamma$ RIIB2 transcript. Primer locations are indicated by the arrows; the name of each primer is shown above the arrow. The length of the PCR products expected from amplification with the given primers is indicated under the brackets. The hybridization probes are indicated by bars under the cDNAs with probe names below the bars.

products have the same hybridization pattern (Fig. 5B-D, lane 2). Occasionally RT-PCR with Fc $\gamma$ RIIA-specific primers yields PCR products of unknown significance which migrate between the 762 bp and 639 bp bands.

(b) Fc $\gamma$ RIIA mRNAs are present in low levels in B lymphocytic cell lines and to a much greater extent in myeloid cells and cell lines. Fc $\gamma$ RIIA mRNAs were not

detected in RPMI 1788 cells, a B lymphocytic cell line, by Northern blot analysis (Fig. 1A, lane 6). Occasionally, Fc $\gamma$ RIIA transcripts were detected by RT-PCR in RPMI 1788 as well as in another B lymphocytic cell line, Raji (Fig. 5A, lanes 5 and 6). Taken together, this data suggests that Fc $\gamma$ RIIA mRNAs are relatively minor transcripts in the B lymphocytic cell lines tested. In contrast, the myeloid cell lines, K562 and U937, express

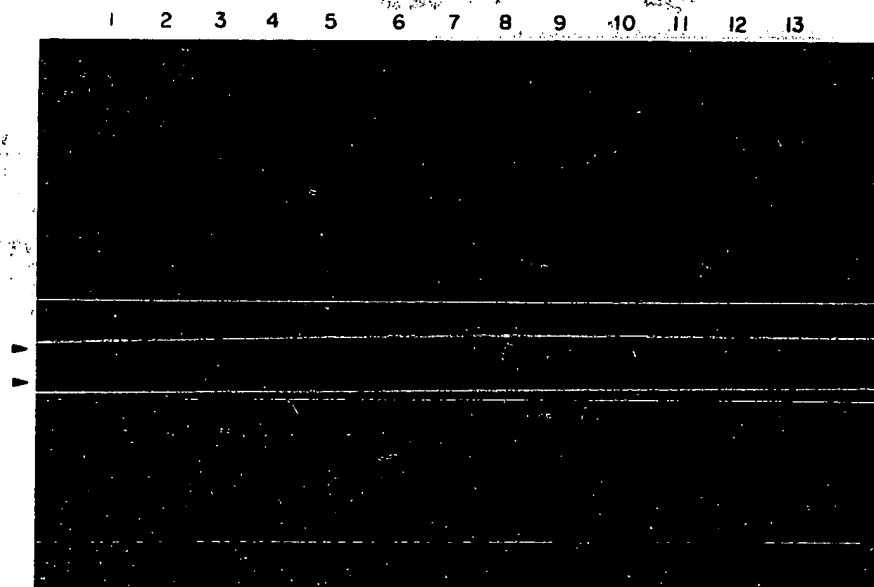
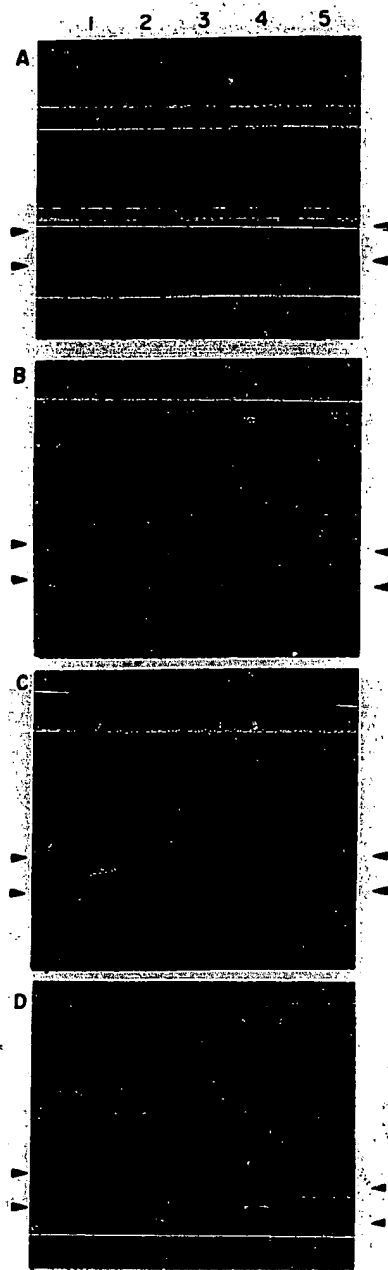
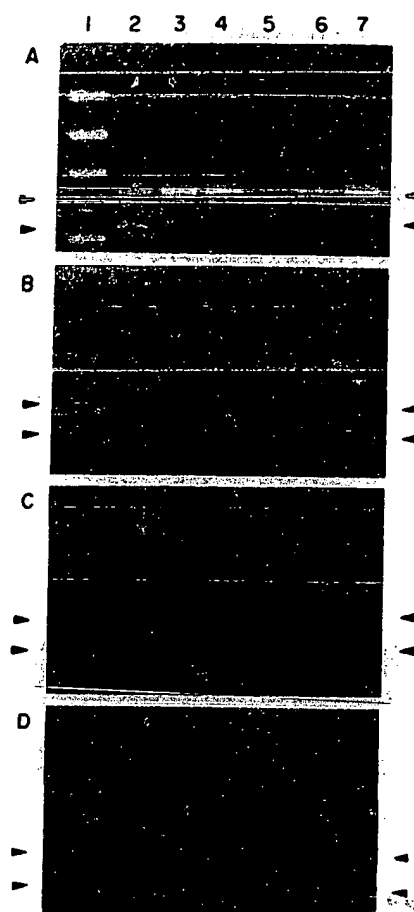


Fig. 3. RT-PCR analysis of platelet RNA with Fc $\gamma$ RIIA-, Fc $\gamma$ RIIB-, and Fc $\gamma$ RIIC-specific primer pairs. The 1.5% agarose gel stained with ethidium bromide is shown. Lanes 3, 4, 7, 8, 11 and 12 are reactions performed with an equal amount of input platelet RNA. Lanes 2, 6 and 10 are reactions performed with no input RNA. Lanes 1 and 13 contain a HaeIII digest of  $\Phi$ X174 as size markers. With the Fc $\gamma$ RIIA-specific primer pair (lanes 2-5), platelet RNA yielded 762 bp and 639 bp bands (lanes 3 and 4), while the HFc3.0 cDNA control yielded the expected 762 bp band (lane 5). Using the Fc $\gamma$ RIIB-specific primer pair (lanes 6-9), there were no PCR products produced in platelet RNA (lanes 7 and 8) while the Fc $\gamma$ RIIB2 cDNA control yielded the expected 795 bp band. The Fc $\gamma$ RIIC-specific primer pair (lanes 10-12) yielded a faint 801 bp band in platelet RNA (lanes 11 and 12). The upper arrowhead indicates the 762 bp band corresponding to the Fc $\gamma$ RIIA1 transcript and the lower arrowhead indicates the 639 bp band corresponding to the Fc $\gamma$ RIIA2 transcript.



**Fig. 4.** RT-PCR analysis of blood cell RNA with Fc $\gamma$ RIIA-specific primers. (A) The 1.5% agarose gel stained with ethidium bromide is shown. Lane 1, HaeIII digest of  $\Phi$ X174 as size markers; Lane 2, platelets; lane 3, granulocytes; lane 4, monocytes; lane 5, Fc $\gamma$ RIIA cDNA HFc3.0. Platelet RNA yielded 762 bp and 639 bp bands and bands of intermediate size. Granulocytes and monocytes yielded a major 762 bp band and a minor 639 bp band. HFc3.0 yielded the expected 762 bp band. (B) The DNA in gel A was transferred to a nylon membrane and hybridized with the oligonucleotide 16M, which hybridizes in the S1 exon of Fc $\gamma$ RIIA. (C) The stripped blot was reprobed with the oligonucleotide TM, specific for the TM region. (D) The stripped blot was reprobed with the oligonucleotide DL, which hybridizes to sequences brought together by the splicing out of the TM exon. The upper arrowhead indicates the 762 bp band corresponding to the Fc $\gamma$ RIIA1 transcript and the lower arrowhead indicates the 639 bp band corresponding to the Fc $\gamma$ RIIA2 transcript. The species between these arrows are uncharacterized, and hybridization to this band is seen with both the TM and DL probes.



**Fig. 5.** RT-PCR analysis and Southern blotting of Fc $\gamma$ RIIA transcripts. (A) The 1.5% agarose gel stained with ethidium bromide is shown. Myeloid (lanes 3 and 4), megakaryocytic (lane 2) and B lymphocytic (lanes 5 and 6) cell RNAs were analyzed. Lane 1, HaeIII digest of  $\Phi$ X174 as size markers; lane 2, platelets; lane 3, K562; lane 4, U937; lane 5, Raji; lane 6, RPMI 1788; lane 7, Fc $\gamma$ RIIA cDNA HFc3.0. Platelet RNA yielded 762 and 639 bp bands. K562 and U937 have 762 bp as their major band and a band at 639 bp. Raji RNA yields a faint 639 bp band. RPMI 1788 RNA yields 762 bp, 639 bp, and intermediate bands. (B) The gel in A was Southern blotted and probed with the oligonucleotide 16M, which hybridizes in the S1 exon of Fc $\gamma$ RIIA. (C) The stripped blot was reprobed with the oligonucleotide TM, which hybridizes to sequences in the TM exon. (D) The stripped blot was reprobed with DL, an oligonucleotide specific for TM-lacking gene products. The upper arrowhead indicates the 762 bp band corresponding to the Fc $\gamma$ RIIA1 transcript; the lower arrowhead indicates the 639 bp band, corresponding to the Fc $\gamma$ RIIA2 transcript.

high levels of Fc $\gamma$ RIIA mRNAs by both Northern blot analysis (Fig. 1A, lanes 1 and 5) and RT-PCR (Fig. 5A, lanes 3 and 4). Fc $\gamma$ RIIA transcripts were also observed in RNA from granulocytes and monocytes by RT-PCR (Fig. 4, lanes 3 and 4).

(c) *Fc $\gamma$ RIIA mRNA coding for a soluble receptor is present at levels comparable to the membrane form in megakaryocytic but not myeloid cells.* We consistently observed that transcripts of the Fc $\gamma$ RIIA gene containing the transmembrane region (Fc $\gamma$ RIIA1) and those

lacking this domain (Fc $\gamma$ RIIa2) were present in comparable amounts in RNA from platelets (Fig. 3, lanes 3 and 4; Fig. 4, lane 2) and HEL cells (Fig. 5, lane 2). We have also observed comparable amounts of these two transcripts in the megakaryocytic cell lines, CHRF-288-11 and Dami (data not shown). In contrast, RT-PCR shows that the prevalence of Fc $\gamma$ RIIa1 and Fc $\gamma$ RIIa2 transcripts differs in myeloid cells and cell lines. The transmembrane form dominates in granulocytes and

monocytes (Fig. 4, lanes 3 and 4), as well as in K562 and U937 cell lines (Fig. 5, lanes 3 and 4).

## (2) Fc $\gamma$ RIIB and Fc $\gamma$ RIIC transcript distribution

(a) Northern blot analysis of Fc $\gamma$ RIIB/IIC transcripts. Fc $\gamma$ RIIB/IIC transcripts were not detected by Northern blot analysis in the megakaryocytic cell lines, HEL, CHRF-288-11 and Dami (Fig. 1B, lanes 1-3). The myeloid cell lines, K562 and U937, and the lymphoid cell line, RPMI 1788, have 1.6 kb Fc $\gamma$ RIIB/IIC transcripts (Fig. 1B, lanes 4-6).

(b) RT-PCR analysis of Fc $\gamma$ RIIB transcripts. RT-PCR analysis was performed with a Fc $\gamma$ RIIB-specific primer pair that brackets the first cytoplasmic exon present in the Fc $\gamma$ RIIB1 transcript and lacking in the Fc $\gamma$ RIIB2 transcript (Fig. 2). These two transcripts of Fc $\gamma$ RIIB were present in myeloid (K562 and U937) and lymphoid (Raji and RPMI 1788) cell lines (Fig. 6A, lanes 4-7). The sizes of the PCR products corresponded to those expected for Fc $\gamma$ RIIB1 (852 bp) and Fc $\gamma$ RIIB2 (795 bp). The C1 oligonucleotide probe, specific for the first cytoplasmic exon, hybridized only to the 852 bp band and not to the 795 bp band or to the control Fc $\gamma$ RIIB2 PCR product as expected (Fig. 6D, lanes 4-8). No Fc $\gamma$ RIIB PCR products lacking the TM exon were observed (Fig. 6C). In contrast, we detected no Fc $\gamma$ RIIB transcripts in HEL RNA (Fig. 6, lane 2) or in platelet RNA (Fig. 3, lanes 7 and 8). Fc $\gamma$ RIIA transcripts were readily amplified from the same RNA preparations, confirming the integrity of the RNA. In the same RT-PCR experiments, the Fc $\gamma$ RIIB primers successfully amplified transcripts from myeloid and lymphoid cell lines as well as from the control Fc $\gamma$ RIIB2 cDNA, confirming PCR performance with these primers. CHRF-288-11 cells had no Fc $\gamma$ RIIB/IIC transcripts by Northern blot analysis (Fig. 1B, lane 2). Faint bands seen in RT-PCR of CHRF-288-11 RNA have the hybridization characteristics of a Fc $\gamma$ RIIB2 transcript (Fig. 6, lane 3).

(c) RT-PCR analysis of Fc $\gamma$ RIIC transcripts. RT-PCR analysis with Fc $\gamma$ RIIC-specific primers indicated a 801 bp band in K562, U937, Raji and RPMI 1788 cells (Fig. 7A, lanes 4-7). This band matches the size predicted by the published cDNA sequence (Stuart *et al.*, 1989). There were also one (Raji and RPMI 1788) or two (K562 and U937) larger PCR products. Hybridization with the 17M probe confirmed the Fc $\gamma$ RIIC origin of all products (Fig. 7B, lanes 4-7). Hybridization of the TM probe to all bands demonstrated that these transcripts contain the transmembrane region (Fig. 7C, lanes 4-7). We detected no Fc $\gamma$ RIIC transcripts in HEL cells by either Northern blot analysis (Fig. 1B, lane 1) or RT-PCR (Fig. 7A, lane 2). Although RT-PCR indicated that low levels of Fc $\gamma$ RIIC transcripts were present in CHRF-288-11 cells (Fig. 7A, lane 3), Northern blot analysis with the Fc $\gamma$ RIIB/IIC-specific oligonucleotide probe did not confirm this finding (Fig. 1B, lane 2). A faint 801 bp band was detected in platelet RNA (Fig. 3A, lanes 11 and 12). This Fc $\gamma$ RIIC band was less than 1/100 the intensity of the Fc $\gamma$ RIIA bands from the same platelet

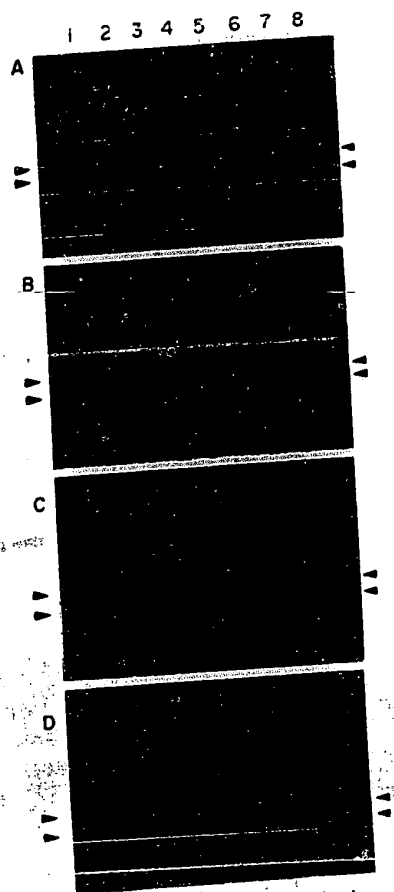


Fig. 6. RT-PCR analysis and Southern blotting of Fc $\gamma$ RIIB transcripts. The Fc $\gamma$ RIIB-specific primer pair was used in RT-PCR with various RNA sources. (A) The 1.5% agarose gel stained with ethidium bromide is shown. Myeloid (lanes 4 and 5), megakaryocytic (lanes 2 and 3) and B lymphocytic (lanes 6 and 7) cell RNAs were analyzed. Lane 1, HaeIII digest of  $\phi$ X174 as size markers; lane 2, HEL; lane 3, CHRF-288-11; lane 4, K562; lane 5, U937; lane 6, Raji; lane 7, RPMI 1788; lane 8, Fc $\gamma$ RIIB2 cDNA control. HEL RNA yielded no PCR products. CHRF-288-11 yielded a faint 795 bp band. K562, U937, Raji and RPMI 1788 all yielded 852 bp and 795 bp bands. (B) The gel in A was Southern blotted and hybridized with 17M, an oligonucleotide specific for the S1 exon in Fc $\gamma$ RIIB/IIC. (C) The stripped blot was reprobed with TM, an oligonucleotide complementary to sequences in the TM exon. (D) The stripped blot was reprobed with the oligonucleotide C1, which hybridizes to sequences in the C1 exon. Arrowheads indicate the positions of the 852 bp and 795 bp band, corresponding to the Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2 transcripts, respectively.

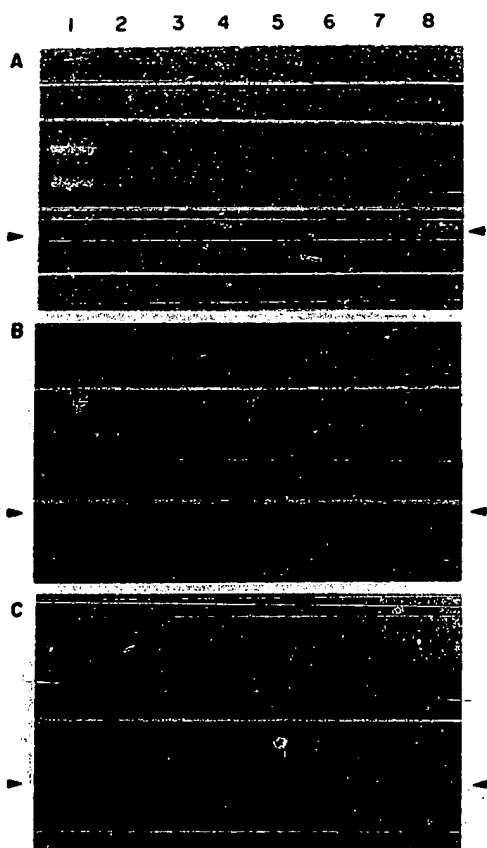


Fig. 7. RT-PCR analysis and Southern blotting of Fc $\gamma$ RIIC transcripts. (A) The 1.5% agarose gel stained with ethidium bromide is shown. Myeloid (lanes 4 and 5), megakaryocytic (lane 2 and 3) and B lymphocytic (lanes 6 and 7) cell RNAs were analyzed. Lane 1, HaeIII digest of  $\phi$ X174 as size markers; lane 2, HEL; lane 3, CHRF-288-11; lane 4, K562; lane 5, U937; lane 6, Raji; lane 7, RPMI 1788. Lane 8 shows the Fc $\gamma$ RIIb2 cDNA used as a PCR control amplified with the Fc $\gamma$ RIIB-specific primer pair. HEL RNA yielded no PCR products. CHRF-288-11 yielded a faint 801 bp band. K562 and U937 yielded a 801 bp band and two upper bands. Raji and RPMI 1788 yielded an 801 bp band and an upper band. (B) The gel seen in A was Southern blotted and probed with 17M, an oligonucleotide that hybridizes to sequences in the S1 exon of Fc $\gamma$ RIIB/IIC. (C) The same blot was stripped and probed with TM, an oligonucleotide complementary to sequences in the TM exon. The arrow indicates the 801 bp band, corresponding to the major Fc $\gamma$ RIIC transcript.

RNA preparation in the same RT-PCR experiment as determined by serial dilution of the Fc $\gamma$ RIIA-specific RT-PCR reaction (data not shown).

### (3) Summary of differential expression

In summary, Fc $\gamma$ RIIA, Fc $\gamma$ RIIB and Fc $\gamma$ RIIC mRNAs are differentially expressed in hematopoietic cells. Megakaryocytic cells predominantly express Fc $\gamma$ RIIA and no Fc $\gamma$ RIIB mRNAs. Fc $\gamma$ RIIa1 and Fc $\gamma$ RIIa2 transcripts are present in comparable amounts. Myelomonocytic cells contain Fc $\gamma$ RIIa1 as the predominant Fc $\gamma$ RIIA transcript, as well as Fc $\gamma$ RIIb1, Fc $\gamma$ RIIb2 and Fc $\gamma$ RIIC transcripts. We have

found that the major transcripts in B lymphocytes are Fc $\gamma$ RIIb1, Fc $\gamma$ RIIb2 and Fc $\gamma$ RIIC. All cells positive by Northern blot analysis with the Fc $\gamma$ RIIB/IIC probe were shown by RT-PCR analysis to have both Fc $\gamma$ RIIB and Fc $\gamma$ RIIC transcripts.

### DISCUSSION

We used a combination of Northern blot analysis and RT-PCR to elucidate Fc $\gamma$ RII transcript distribution in hematopoietic cells. Delineation of the specific Fc $\gamma$ RII genes expressed in the megakaryocytic lineage has not been reported. The distribution of the human Fc $\gamma$ RIIa1 and Fc $\gamma$ RIIa2 transcripts and the Fc $\gamma$ RIIb1 and Fc $\gamma$ RIIb2 transcripts was also unknown. Brooks *et al.* (1989) and Stuart *et al.* (1989) examined Fc $\gamma$ RII transcripts on Northern blots using RNA from a variety of hematopoietic cells and cell lines; however, megakaryocytic cells were not examined. Stuart *et al.* (1989) were unable to individually distinguish the Fc $\gamma$ RIIA, Fc $\gamma$ RIIB or Fc $\gamma$ RIIC mRNAs and neither group was able to characterize the distribution of Fc $\gamma$ RIIa1, Fc $\gamma$ RIIa2, Fc $\gamma$ RIIb1 and Fc $\gamma$ RIIb2 transcripts. We now demonstrate the differential expression of Fc $\gamma$ RII transcripts including Fc $\gamma$ RIIa1, Fc $\gamma$ RIIa2, Fc $\gamma$ RIIb1 and Fc $\gamma$ RIIb2 in megakaryocytes, myeloid cells and lymphoid cells.

Northern blots and gene-specific hybridization probes traditionally have been used to demonstrate the presence of specific mRNAs. One disadvantage of this technique is the difficulty of distinguishing mRNAs arising from highly homologous genes. This can be overcome using RT-PCR with gene-specific primer pairs. It is also difficult to resolve similarly-sized transcripts by Northern blot analysis; for example, Fc $\gamma$ RIIa1 and Fc $\gamma$ RIIa2 transcripts, differing by the presence or absence of the 123 bp transmembrane exon, are not resolved. However, these transcripts can be easily distinguished by using primers bracketing the transmembrane exon in RT-PCR. Another advantage of RT-PCR is its extreme sensitivity. This sensitivity can also be a disadvantage, since very low abundance transcripts of questionable significance may be detected. Therefore, Northern blot analysis in conjunction with RT-PCR provided a means to accomplish a comprehensive study of Fc $\gamma$ RII transcript distribution in hematopoietic cells.

Using this strategy, we demonstrated that Fc $\gamma$ RIIA is the predominant Fc $\gamma$ RII mRNA in both primary cells (platelets) and cell lines (HEL, CHRF-288-11 and Dami) of the megakaryocytic lineage. These cell lines provide a model system to study Fc $\gamma$ RIIA function, since Fc $\gamma$ RII is the only Fc $\gamma$  receptor class expressed (King *et al.*, 1992; data not shown) and here we show that Fc $\gamma$ RIIA mRNA predominates in this lineage.

Megakaryocytic cells express transcripts of Fc $\gamma$ RIIA differing by the presence or absence of the TM region. In other work, we have demonstrated that these transcripts result from alternative splicing of the TM exon (Walterhouse *et al.*, 1988; Rappaport *et al.* 1993). The transcript lacking the TM exon, Fc $\gamma$ RIIa2, may



code for a potentially soluble form of Fc $\gamma$ RIIA. A soluble receptor that arises by alternative splicing of the TM exon is distinct from one that arises by proteolytic cleavage of a membrane transcript (eg. murine Fc $\gamma$ RII $\beta$ ) by phosphatidylinositol-phospholipase C cleavage of a phosphatidylinositol-anchored form (e.g. human Fc $\gamma$ RIIIB). Splicing out of a transmembrane exon and retention of the cytoplasmic domain occurs in the production of other members of the immunoglobulin supergene family, including HLA-A24 (Krangel, 1986), HLA-DQB (Briata *et al.*, 1989), CD8 (Giblin *et al.*, 1989), the GM-CSF receptor (Ashworth and Kraft, 1990), the IL-4 receptor (Mosley *et al.*, 1989) and the IL-7 receptor (Goodwin *et al.*, 1990). In megakaryocytic cells, the Fc $\gamma$ RIIa1 and Fc $\gamma$ RIIa2 transcripts appear to be equally represented. In contrast, the Fc $\gamma$ RIIa1 transcript predominates in myeloid cells. These observations suggest that splicing of the transmembrane exon is under lineage-specific control. We hypothesize that megakaryocytes and platelets are a major source of soluble Fc $\gamma$ RIIA protein. A soluble Fc $\gamma$ RIIA receptor can be predicted to compete with the integral membrane Fc $\gamma$ RIIA receptor for binding IgG-containing immune complexes and, thus, play an important regulatory role in immune function.

A polymorphism has been defined in the extracellular domain of Fc $\gamma$ RIIA that influences the relative binding of anti-Fc $\gamma$ RII monoclonal antibody, 41H16 (Clark *et al.*, 1989; Gosselin *et al.*, 1990). This allotypic difference in the binding of 41H16 has been observed with platelet, neutrophil and monocyte Fc $\gamma$ RII but not with B lymphocyte Fc $\gamma$ RII, suggesting that Fc $\gamma$ RIIA is not expressed in B lymphocytes (Gosselin *et al.*, 1990). Thus, our Fc $\gamma$ RIIA transcript distribution data is in agreement with the available information regarding Fc $\gamma$ RII protein distribution.

In contrast to Fc $\gamma$ RIIA transcripts, all Fc $\gamma$ RIIB and Fc $\gamma$ RIIC transcripts in all blood cell lineages examined contained the TM region. We were able to distinguish the Fc $\gamma$ RIIB transcripts, Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2, which differ in the presence or absence of the first cytoplasmic exon. Both transcripts are present in lymphocytic cell lines (Raji and RPMI 1788), as well as in myeloid cell lines (U937 and K562). This is in contrast to the expression of the murine forms homologous to human Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2. In the mouse, Fc $\gamma$ RIIB2, which lacks the first cytoplasmic exon, is found only in cells of the myeloid lineage where it is capable of endocytosis of immune complexes. Murine Fc $\gamma$ RIIB1 which retains the first cytoplasmic exon is present in lymphocytic cells and does not function in endocytosis. It has been suggested that the insertion of sequences in the cytoplasmic tail of the murine Fc $\gamma$ RIIB1 protein interferes with coated pit-mediated endocytosis (Miettinen *et al.*, 1989). In other work, human Fc $\gamma$ RIIa1, Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2 cDNAs have been transfected into COS-1 cells (Indik *et al.*, 1992a, b). All three proteins were capable of binding antibody-coated erythrocytes; however, only cells transfected with Fc $\gamma$ RIIA were able to phagocytose the antibody-coated erythrocytes.

These results suggest that if Fc $\gamma$ RIIB1 and/or Fc $\gamma$ RIIB2 mediate phagocytosis in the macrophage, additional molecules beyond those found in the COS cell may be required. The function of both forms of human Fc $\gamma$ RIIB in both myeloid and lymphoid cells remains unknown. We speculate that the two forms may function in different signaling pathways in the same cell type, in contrast to the case of the murine homologues.

These studies reveal the existence of Fc $\gamma$ RII transcripts, Fc $\gamma$ RIIa1, Fc $\gamma$ RIIa2, Fc $\gamma$ RIIB1, Fc $\gamma$ RIIB2, and Fc $\gamma$ RIIC, predicting at least five distinct proteins differentially expressed in platelets, neutrophils, monocytes and B lymphocytes. We have made progress in deciphering Fc $\gamma$ RII expression at the mRNA level. The challenges for future work are to decipher the regulatory mechanisms underlying differential expression and to examine the functional consequences resulting from the structural diversity.

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